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CA 2365290 A1 2000/09/08

(21) 2 365 290

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2000/02/29
(87) Date publication PCT/PCT Publication Date: 2000/09/08
(85) Entrée phase nationale/National Entry: 2001/09/04
(86) N° demande PCT/PCT Application No.: EP 2000/001682
(87) N° publication PCT/PCT Publication No.: 2000/051578
(30) Priorité/Priority: 1999/03/03 (199 09 115.3) DE

(51) Cl.Int.^{7}/Int.Cl.^{7} A61P 11/00, A61K 31/16

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(54) Titre : UTILISATION DE CERAMIDES POUR LE TRAITEMENT DE LA MUCOVISCIDOSE
(54) Title: USE OF CERAMIDES FOR THE TREATMENT OF CYSTIC FIBROSIS

(57) Abrégé/Abstract:
The invention relates to the use of ceramides and/or substances which contain ceramides as a building block. Said ceramides and substances are used for the treatment of cystic fibrosis. The ceramides are especially C2 and/or C6 ceramides. The invention also relates to the use of the above-mentioned substances for the treatment of diseases which are linked to a disturbed regulation of transport processes at membranes.

ABSTRACT

USE OF CERAMIDES FOR THE TREATMENT OF CYSTIC FIBROSIS

The invention relates to the use of ceramides and/or the use of substances containing ceramides as the building block for the treatment of cystic fibrosis. The ceramides are in particular C2 and/or C6 ceramides. The invention also covers the use of the aforementioned substances for the treatment of diseases, which are linked with a disturbed regulation of transport processes at membranes.

DESCRIPTION

USE OF CERAMIDES FOR THE TREATMENT OF CYSTIC FIBROSIS

[001] The invention relates to the use of ceramides, derivatives of ceramides and/or precursors of ceramides. The invention more particularly relates to the use of these substances in conjunction with the treatment of cystic fibrosis.

[002] Cystic fibrosis is the most frequently encountered hereditary metabolic disease with several hundred thousand deaths annually throughout the world making it the most frequently encountered lethal, genetic disease. The frequency of occurrence of this disease regionally differs and in Europe one out of 2,000 newborn babies are affected.

[003] Cystic fibrosis is a recessive autosomal metabolic disorder, which is linked with a generalized malfunction of exocrine glands. As a result of an increased electrolyte content of the secretion of sweat glands liquid and electrolyte losses occur. In addition, the viscosity of the secretions is increased, so that serious complications arise in the area of the respiratory tracts and digestive tract with secondary cystogenesis. The disease gives rise to a considerable reduction of life expectancy.

[004] One of the pathophysiological mechanisms in cystic fibrosis (CF) is a disturbed regulation of mainly epithelial Cl^-, Na^+ and K^+ channels. Chloride channels are in particular affected, transporting Cl^- ions out of the cell, namely outwardly rectifying chloride channels or ORCC.

[005] The lack of activatability of these chloride channels as a result of the disease and disturbed regulation of Na^+ and water-resorbing transport processes leads to a reduced epithelial secretion of liquids and bicarbonate. This leads to viscous secretions in the lungs, pancreas and intestine of affected patients leading to an obstruction of respiratory tracts, pancreatic ducts and the intestine. This is favourable to pulmonary infections and leads to malabsorption.

[006] Cystic fibrosis is caused by numerous different mutations of the CFTR gene, which for chloride channels and/or regulators of chloride channels codes Na^+ and K^+ channels. The mutations lead to a disturbed transepithelial transport with reduced Cl^- and HCO_3^- secretion and increased Na^+ resorption.

[007] The problem of the invention is to make available substances and processes positively influencing the aforementioned processes disturbed in the case of CF patients. For example it is intended to activate liquid secretion in the epithelia of CF patients and in this way at least improve

pathogenesis.

[008] This problem is solved by the use of ceramides and/or substances with a ceramide building block according to claim 1 and the dependent claims 2 to 4. The problem is also solved by the use of biological precursor molecules according to claim 5 and activators according to claim 6. Pharmaceutical compositions containing at least one of these active substances are claimed in claims 7 to 12. Claims 13 to 17 relate in general terms to the use of said substances in the treatment of diseases, which are linked with disturbed transport processes. By reference the wording of all of the claims is made into part of the content of the present description.

[009] The chloride channels in cells of healthy people, but not in those of CF patients, can be activated by cAMP (cyclic adenosine monophosphate) [Rich et al, Nature 347: 358-363, 1990]. According to the prior art it was to be assumed that it would not be possible to activate the chloride channels in the cells of CF patients. However, this defect gives rise to the lack of liquid secretion of the cells, which is an important cause of the symptoms of cystic fibrosis. It has recently been possible to show that the outwardly rectifying chloride channels (ORCC channels) in cells from healthy patients can be activated by ceramides [Szabo et al, Acad. Sci. USA, 95(11): 6169-6174, 1998].

[010] Surprising results forming the basis for the present invention show that also the chloride channels in lymphocytes of CF patients can be activated by ceramides, although the regular activatability by cAMP (cyclic adenosine monophosphate) is disturbed. The channels activated by ceramides bring about a liquid secretion of the cells from CF patients, so that through the use of ceramides the symptoms of cystic fibrosis are improved.

[011] As is known ceramides are endogenic substances, which in particular occur in the brain substance and myelin of the central nervous system. They are lipophilic amides. Ceramides occur in the organism as choline phosphate esters or as glycosides. Ceramides form the building blocks of choline phosphate esters, also known as sphingomyelins, or glycosides occurring as so-called cerebrosides, gangliosides and sulphatides. The two groups of substances with ceramides as building blocks are called sphingolipids. From the chemical standpoint they are lipids, which in place of glycerin (in the case of fats and oils) contain as the alcohol component sphingosine (4-sphingogenin), which does not freely occur in nature.

[012] The invention relates to the use of ceramides and/or other of the aforementioned substances for the treatment of cystic fibrosis. Through the administration of an effective quantity of such substances it is preferably possible to stimulate liquid secretion in the epithelia of patients.

Particular reference is made to the pulmonary, pancreatic and intestinal epithelia of CF patients. The invention also covers the treatment of cystic fibrosis by the administration of ceramides and/or the other aforementioned substances, as well as the use of all these substances for the production of medicaments, particularly those for the treatment of cystic fibrosis.

[013] The ceramides or substances with a ceramide building block used according to the invention are, in a preferred embodiment, longer chain ceramides, such as e.g. the C12 ceramide or derivatives thereof, which as frequently naturally occurring substances can be directly isolated from cell material. The ceramides/substances used can also be obtained by enzymatic treatment of corresponding precursors isolatable from cell material. In a further embodiment of the invention use is made of ceramides/substances, which are obtained with the aid of molecular biological methods which are known to the expert. In a particularly preferred embodiment use is made according to the invention of synthetically produced ceramides/substances, more particularly the C2 or C6 ceramides/substances with said ceramides as the building block.

[014] According to the invention it is also possible to use derivatives of ceramides, which are chemically or biologically modified in their ceramide building blocks. With respect to cystic fibrosis, these derivatives evolve a similar and preferably better action than ceramides and/or have similar or better pharmaceutical characteristics. These can in particular be e.g. slower decomposition rates of the active substance in the body or better absorption rates in the cells.

[015] In a preferred embodiment, the substances used according to the invention are biological precursor molecules of ceramides or substances with a ceramide building block which, e.g. through an enzymatic cleaving by sphingomyelinases, are transformed in the cell into active ceramides.

[016] According to a further preferred embodiment of the invention, the substances used according to the invention act metabolically leading to the formation of ceramides. In particular, use is made of activators of sphingomyelinases, which bring about the cleavage of precursor molecules to active ceramides.

[017] A further possible point of attack for activators are cell components, preferably enzymes, which follow the ceramides in the signal chain. In particular, consideration can be given to kinases, which are activated by ceramides.

[018] Apart from the direct administration of the aforementioned active substances, the invention also covers the introduction of nucleic acids,

which code for these substances.

[019] The invention also covers pharmaceutical compositions, which contain at least one ceramide and/or the other said substances and preferably additionally a pharmaceutically acceptable carrier. As to whether use is made of a pharmaceutical carrier and optionally which carrier, is dependent on the medicament administration form.

[020] The administration of all said substances or the pharmaceutical compositions according to the invention can take place systemically. In a preferred embodiment the pharmaceutical composition is administered via the digestive tract. Particular preference is given to administration by inhalation, the active substance being rapidly and directly introduced into the lung. This administration form is particularly appropriate in the treatment of cystic fibrosis, because with this disease there are serious complications with respect to the respiratory organs.

[021] Also in the case of other diseases and illnesses linked with a disturbed regulation of molecular transport processes, preferably at membranes, a treatment with ceramides and/or the other indicated substances can lead to positive results. Therefore the invention covers the use of all these substances in the treatment of such diseases. Preferably the transport processes in the invention take place at ion channels, particularly on outwardly rectifying (from a cell or cell organelle) ion channels, preferably chloride channels.

[022] The described and further features of the invention can be gathered from the following description of preferred embodiments in conjunction with the subclaims and example. The individual features can be implemented singly or in combination with one another. In the drawings show:

Fig. 1 Influencing of the chloride channels in normal lymphocytes (A, B) and in CF lymphocytes (C, D) by cAMP.

Fig. 2 Influencing of chloride channels in CF lymphocytes by ceramide.

Fig. 3 Influencing of chloride channels in normal lymphocytes (A, B) and CF lymphocytes (C, D) by tyrosine kinase Lck^{56}.

Example

[023] The surprising stimulating action of C2/C6 ceramide on chloride channels could be proved in the following experiments. In these experiments the known patch-clamp technique was used. The latter makes it possible to detect the conductivity on biological membranes. Reference is made in this

connection to the document of Szabo et al, Acad. Sci. USA, 95(11): 6169-6174, 1998, whose content in this connection is made into part of the content of the present description.

[024] As is known a micropipette is used to exert suction action on a membrane and by underpressure sealing occurs on the edge of the micropipette. The membrane fixed in this way as part of an intact cell or as an isolated membrane portion is immersed in a suitable electrolyte bath and the conductivity between the latter and the electrolyte solution within the pipette is measured (patch-clamp electrode). By adding different agents to the electrolyte solutions the opening state of ion channels of the membrane can be influenced. Such an influencing can be detected by a sudden conductivity change.

[025] In the present case T-lymphocytes of normal persons and patients suffering from cystic fibrosis (CF) were isolated, introduced into a perfusion chamber and over them was allowed to flow an electrolyte solution (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM hepes/NaOH, pH 7.4). Under the inverse microscope the patch-clamp electrodes (filled with 160 mM Cs-glutamate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 4 Na_2ATP, 10 mM hepes/NaOH, pH 7.2) was brought to the cell membrane and by the sucking in of the cell membrane a link was produced with the intracellular space (whole cell patch clamp) or the conductivity of the sucked membrane patch was measured (cell attached patch clamp). Thus, a continuous registration of the cell membrane conductivity was possible. Following a control period the cells were stimulated either in extracellular manner with cAMP (200 µM), in extracellular with C2 ceramide (50 µM) or in intracellular manner with tyrosine kinase Lck^{56} (10 U/ml) and the conductivity was continuously measured.

[026] The electrodes were connected by means of suitable preamplifiers to a patch-clamp amplifier (EPC-9) with the aid of which the potential between the patch-clamp electrode and a reference electrode was varied in the bath from -100 mV to +100 mV. The flows over the cell membrane between the patch-clamp electrode and the reference electrode were recorded with the aid of the patch-clamp amplifier. The choice of solutions permitted an exclusive analysis of flows through chloride channels.

[027] The curve traces shown in the individual parts of figs. 1 and 3 reflect the conductivity change (whole cell) over a period of several minutes following the administration of the substances in question. The data shown in fig. 2 represent the conductivity of administration of ceramide (cell attached) and 7 minutes following ceramide administration. The administration of cAMP and Lck^{56} took place in the pipette, so that from the curves of figs. 1 and 3, which were recorded at different times, it is not

possible to directly derive the kinetics of activation of chloride channels. This time dependent can be attributed to the diffusion of the supplied substances within the pipette and is consequently dependent on the method chosen. What is mainly decisive for the interpretation is the end point of the conductivity change, i.e. the top curve in each case.

[028] Ceramide was applied outside the pipette. Therefore diffusion played no part in the test, whose results are represented in fig. 2. In the representation chosen only the control value (without ceramide) and the modified conductivity after 7 minutes incubation with ceramide are plotted.

[029] As shown in fig. 1, the administration of cAMP, as expected, led to an activation of the Cl^- flow in normal lymphocytes (figs. 1 A, B), but not in CF lymphocytes (fig. 1 C, D). Thus, in fig. 1 A, C, D the calibration curves determined with time intervals are in each case superimposed, but not in fig. 1 B. As opposed to this and surprisingly ceramide in CF lymphocytes (fig. 2) led to an activation of chloride channels (CF curve before and curve after administration). In addition, the addition of tyrosine kinase Lck^{56}, which is known to be activatable by ceramides, led to an activation of chloride channels in normal lymphocytes (fig. 3 A, B) and in CF lymphocytes (fig. 3 C, D). The curves are superimposed in figs. 3 A and C, but not in figs. 3 B and D, which is in opposition to the results with cAMP.

[030] These results clearly show that the defect in CF cells, i.e. the disturbed activatability of chloride channels, can be positively influenced by the use of ceramides according to the invention.

CLAIMS

1. Use of at least one ceramide and/or at least one substances containing a ceramide as the building block for the preparation of a pharmaceutical composition for the treatment of cystic fibrosis.

2. Use according to claim 1, characterized in that the ceramide is a C2 or a C6 ceramide or the substance contains such a ceramide as the building block.

3. Use according to claim 1 or 2, characterized in that the ceramide and/or the substance with the ceramide component is isolated from biological cell material, particularly from molecular biologically modified cell material.

4. Use according to claim 1 or 2, characterized in that the ceramide and/or the substance with the ceramide building block is synthetically produced.

5. Use of at least one biological precursor of a ceramide or a substance containing a ceramide as the building block for the preparation of a pharmaceutical composition for the treatment of cystic fibrosis.

6. Use of at least one activator, in particular at least one biological activator, which has an activating action on a ceramide, on a substance containing a ceramide as the building block, or on a biological precursor of said ceramide or said substance, for the preparation of a pharmaceutical composition for the treatment of cystic fibrosis.

7. Pharmaceutical composition comprising an effective quantity of at least one ceramide and preferably a pharmaceutical carrier.

8. Pharmaceutical composition according to claim 7, characterized in that the ceramide is a C2 or a C6 ceramide.

9. Pharmaceutical composition according to claim 7 or 8, characterized in that the ceramide is isolated from biological cell material, particularly from molecular biologically modified cell material.

10. Pharmaceutical composition according to claim 7 or 8, characterized in that the ceramide is synthetically produced.

11. Pharmaceutical composition comprising an effective quantity of at least one biological precursor of a ceramide or a substance containing a ceramide as the building block and preferably at least one pharmaceutical carrier.

12. Pharmaceutical composition comprising an effective quantity of at least one activator, preferably at least one biological activator having an

activating action on a ceramide, on a substance containing a ceramide as the building block or on a biological precursor of said ceramide or said substance and preferably at least one pharmaceutical carrier.

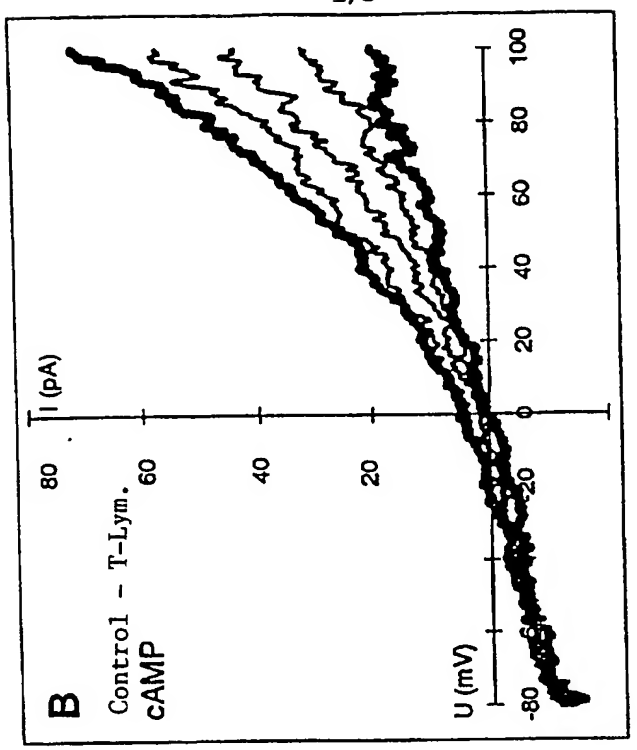
13. Use of at least one ceramide and/or at least one substance containing a ceramide as the building block for the preparation of a pharmaceutical composition for the treatment of diseases linked with a disturbed regulation of transport processes at membranes.

14. Use of at least one biological precursor of a ceramide or a substance containing a ceramide as the building block for the preparation of a pharmaceutical composition for the treatment of diseases linked with a disturbed regulation of transport processes at membranes.

15. Use of at least one activator, particularly at least one biological activator having an activating action on a ceramide, on a substance containing a ceramide as the building block, or on a biological precursor of said ceramide or said substance for the preparation of a pharmaceutical composition for the treatment of diseases linked with a disturbed regulation of transport processes at membranes.

16. Use according to one of the claims 13 to 15, characterized in that the transport processes relate to ion channels, particularly outwardly rectifying chloride channels.

17. Use according to one of the claims 13 to 16, characterized in that the ceramide is a ceramide having a feature according to one of the claims 2 to 4.


B
Control - T-Lym.
cAMP
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60
40
20
U (mV)
100
80
60
40
20
0
-20
-40
-60
-80

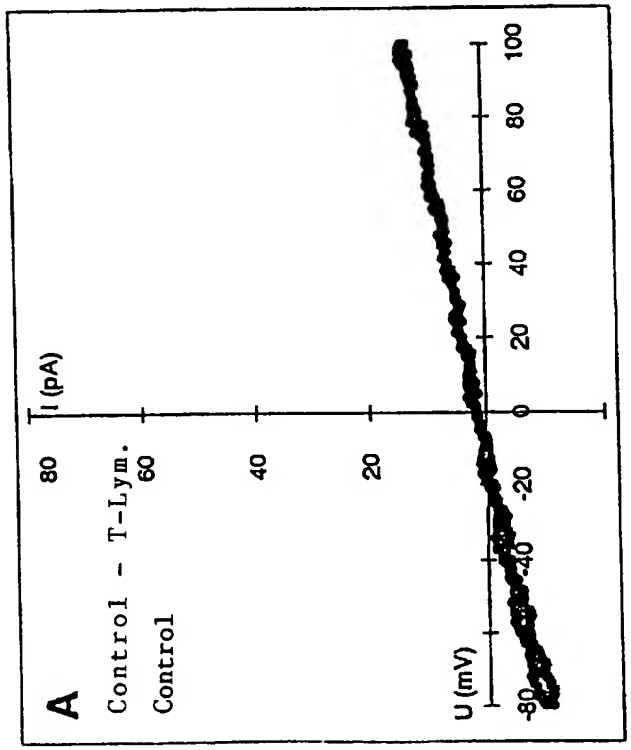
A
Control - T-Lym.
Control
I (pA)
80
60
40
20
U (mV)
100
80
60
40
20
0
-20
-40
-80

FIG. 1A

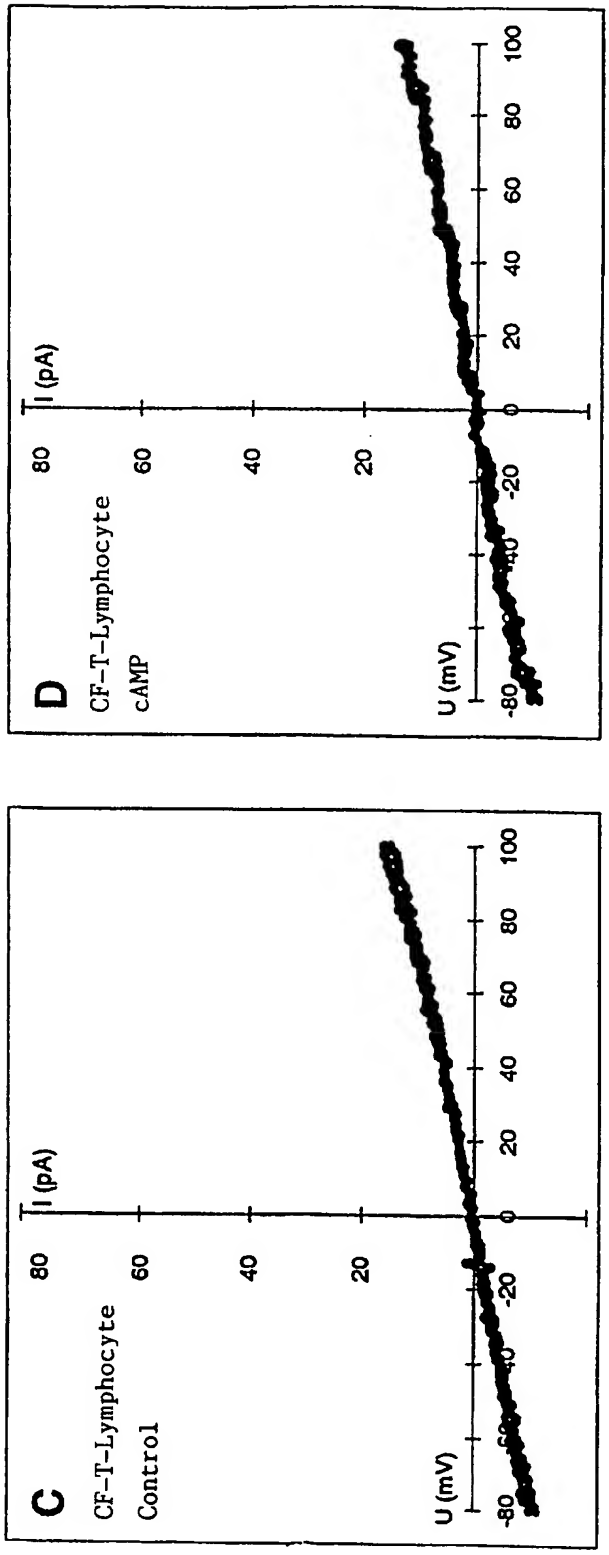
D
CF-T-Lymphocyte
cAMP
I (pA)
U (mV)
C
CF-T-Lymphocyte
Control
I (pA)
U (mV)

FIG. 1B

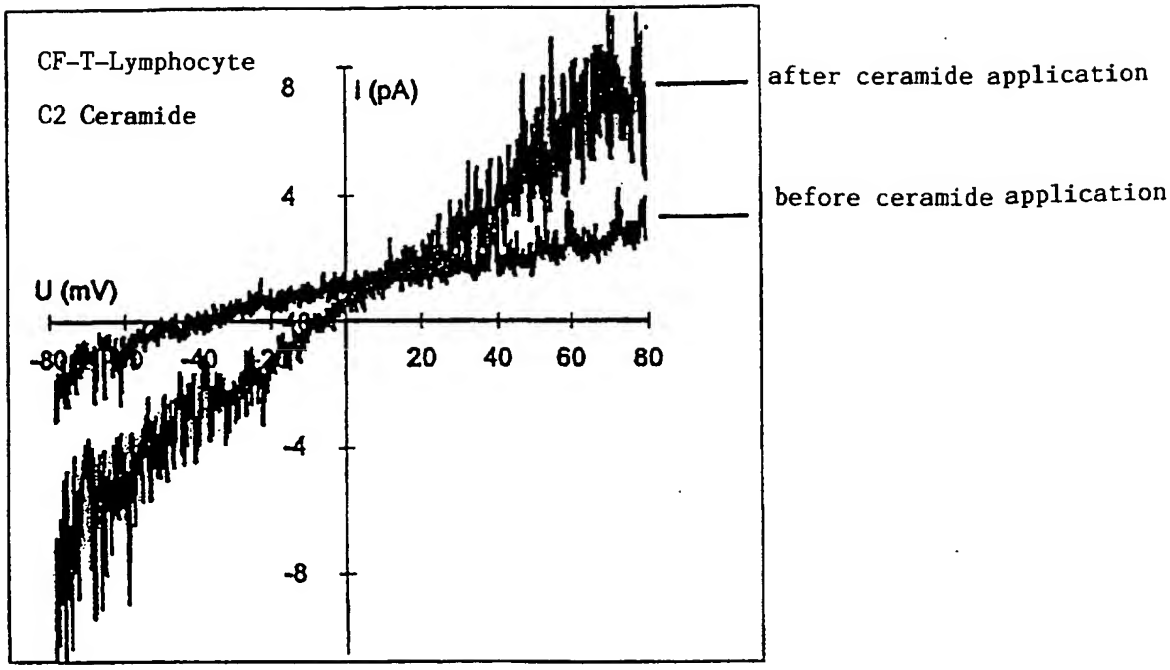
CF-T-Lymphocyte
C2 Ceramide
I (pA)
8
4
-4
-8
U (mV)
-80
-40
20
40
60
80
after ceramide application
before ceramide application

FIG. 2

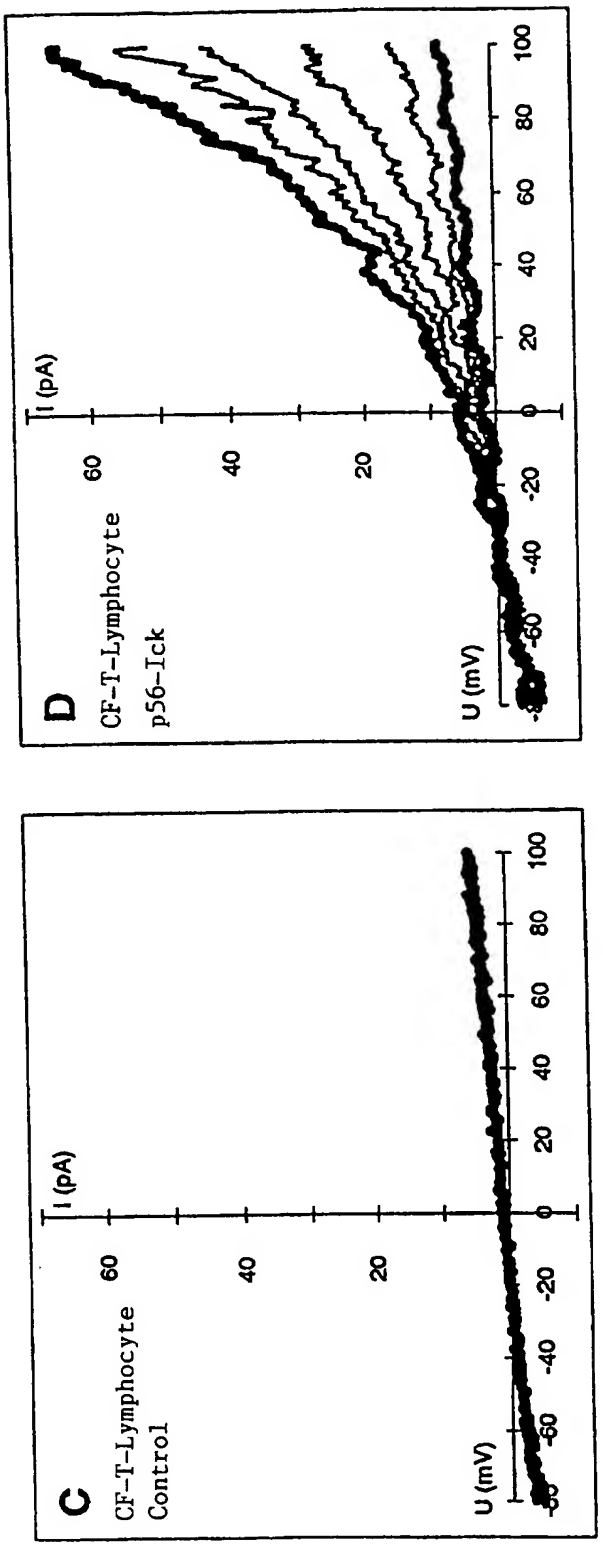
D
CF-T-Lymphocyte
p56-Lck
I (pA)
60
40
20
U (mV)
100
80
60
40
20
0
-20
-40
-60
C
CF-T-Lymphocyte
Control
I (pA)
60
40
20
U (mV)
100
80
60
40
20
0
-20
-40
-60

FIG. 3B

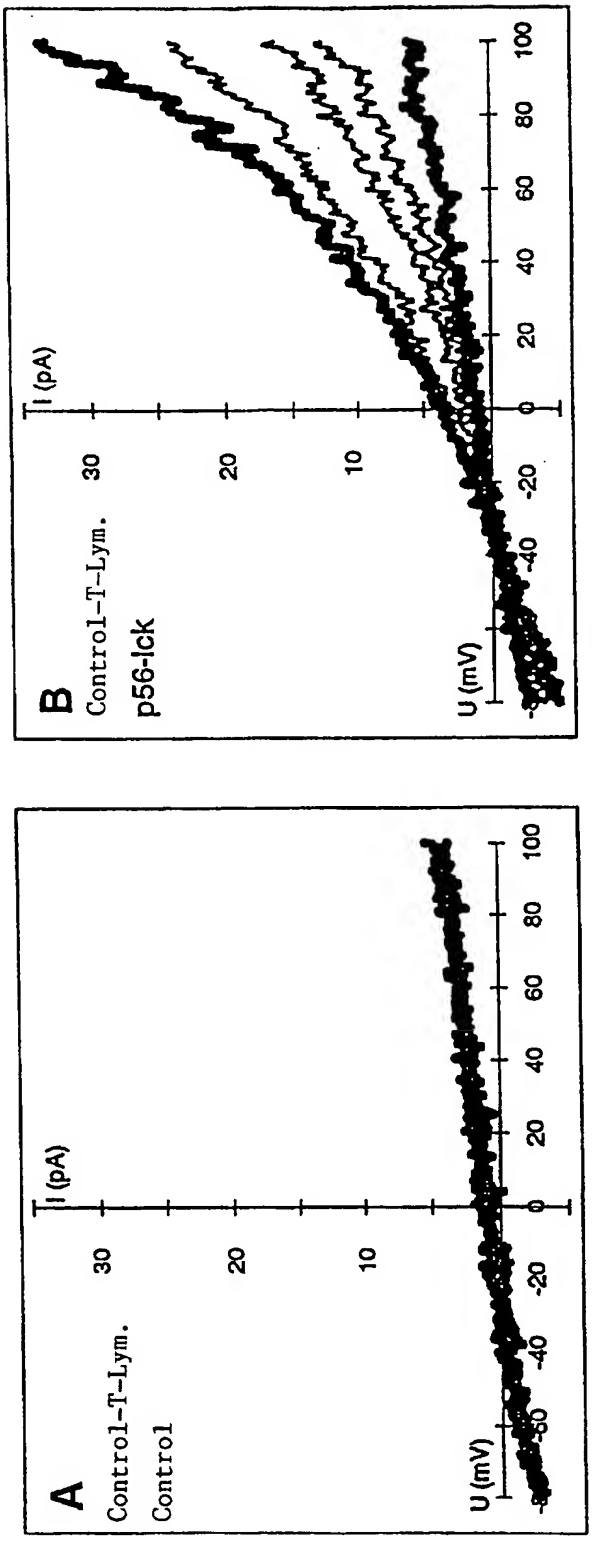
B
Control-T-Lym.
p56-lck
I (pA)
30
20
10
0
-20
-40
U (mV)
100
80
60
40
20
0
A
Control-T-Lym.
Control
I (pA)
30
20
10
0
-20
-40
-60
U (mV)
100
80
60
40
20
0

FIG. 3A

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number
WO 01/72701 A1

(51) International Patent Classification[7]: C07C 321/00

(21) International Application Number: PCT/US01/09894

(22) International Filing Date: 28 March 2001 (28.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/192,719 28 March 2000 (28.03.2000) US

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

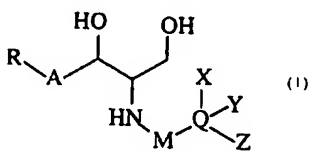
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/72701 A1

(54) Title: CERAMIDE DERIVATIVES AND METHOD OF USE



(1)

(57) Abstract: A ceramide derivative of the formula (I).

CERAMIDE DERIVATIVES AND METHOD OF USE

Field of the Invention

The invention relates to ceramide derivatives, pharmaceutical compositions comprising the ceramide derivatives, and therapeutic uses of the ceramide derivatives as agents against cancer, metabolic diseases such as diabetes, inflammatory conditions and viral infections.

Background of the Invention

Ceramides are a class of naturally occurring sphingolipids normally formed by intracellular hydrolysis of sphingomyelin, and are found in all animal cells. Sphingolipid metabolites participate in signal transduction and cell regulation possibly as first or second messengers. Although neither the direct targets nor the mechanisms of action of ceramides are fully understood, there are several pieces of evidence which suggest that ceramides play an important role as a regulatory component of apoptosis induced by tumor necrosis factor-alpha (TNF-α), Fas ligand, ionizing radiation, and chemotherapeutic agents. Intracellular ceramide is elevated during the induction of apoptosis in a wide variety of cellular systems and addition of cell-permeable ceramide analogs induces apoptosis in many cell lines, providing evidence that ceramide generation plays a direct role in the apoptotic response.

Apoptosis describes a programmed series of events resulting in cell death by fragmentation into membrane-bound particles; these particles are then phagocytosed by other cells (see, e.g., Stedman's Medical Dictionary (Illustrated)). Cells typically undergo apoptosis in physiologically determined circumstances such as the elimination of self-reactive T cells, the death of cells (e.g., neutrophils) with short half-lives, involution of growth factor-deprived cells, morphogenetic cell death during embryonic development and the deaths of cellular targets of cell-mediated cytotoxicity (see, e.g., J. Cohen, *Immunol. Today*, 14(3):126-130 (1993)).

Cells undergoing apoptosis can break up into apoptotic bodies, which are cellular fragments that retain their membranes and are able to regulate their osmotic pressures. Unlike necrotic cells, there is usually no leakage of cellular contents and hence, no invocation of an inflammatory response. Apoptotic cells typically have disrupted plasma membranes and condensed, disrupted nuclei. Nuclear chromatin in these cells is fragmented randomly between nucleosomes, as the result of endonuclease activation during apoptosis.

Although transcription in apoptotic cells ceases, cell death occurs more rapidly than would be expected from the cessation of transcription alone. This indicates that cellular processes in addition to transcription termination are likely to be involved in apoptosis. Gene expression itself may actually be required for the occurrence of the morphological changes associated with apoptosis (see, e.g., J. Cohen, *supra*). Alternatively, inhibition of transcription termination may itself induce apoptosis. Furthermore, apoptosis of some cells does not appear to be affected one way or the other by the inhibition of protein synthesis. Expression of the bcl-2 oncogene, for example, can inhibit the apoptosis otherwise induced by different stimuli, and may thereby contribute to cancer development. Accordingly, inhibition of bcl-2 expression may be required to induce apoptosis (see, e.g., J. Marx, *Science* 259: 760-761 (1993); J. Cohen, *supra*; G. Williams and C. Smith, *Cell* 74:777 (1993); M. Barinaga, *Science* 259: 762 (February 5, 1993)). C-myc protein is known to stimulate cell proliferation; however, it may also stimulate apoptosis in the absence of additional proliferative stimuli. p53, which is thought to suppress tumor growth, may also stimulate apoptosis. C-fas, a transmembrane protein homologous to Tumor Necrosis Factor (TNF), can also induce apoptosis, as can TNF itself.

TNF is a monokine protein produced by monocytes and macrophages. There are two known structurally and functionally related TNF proteins, TNF-α and TNF-β, both of which bind to the same cell surface receptors. Binding to these receptors by TNF leads to the activation of multiple signal transduction pathways, including the activation of sphingomyelinase (see, e.g., M.

Raines et al., *J. Biol. Chem.* 268 (20):14572 (1993); L. Obeid et al., *Science* 259:1769 (March 12, 1993); H. Morishige et al., *Biochim. Biophys. Acta.* 1151:59 (1993); J. Vilcek and T. Lee, *J. Biol. Chem.* 266 (12):7313 (1991); Dbaibo et al., *J. Biol. Chem.* 268 (24):17762 (1993); R. Kolesnik, *Trends Cell. Biol.* 2:232 (1992); J. Fishbein et al., *J. Biol. Chem.* 268 (13):9255 (1993)).

In general, apoptosis occurs in two phases, an initial commitment phase followed by an execution phase resulting in the condensation of nuclear chromatin, fragmentation of DNA, and alterations to the cell membrane. Caspases, a family of cysteine proteases, play a critical role in the execution phase of apoptosis; they participate in a cascade that is triggered in response to proapoptotic signals and in cleavage of intracellular substrates, resulting in disassembling cells. Based on their function in the cascade, caspases can be grouped into two categories, initiators and effectors. Different initiator caspases such as caspase 8 and 9 mediate distinct sets of signals. Caspase 8 binds with the death effector domain DED of the Fas receptor and a cofactor FADD (Fas-associated protein with death domain). The activation of caspase 9 requires several cofactors such as cytochrome c, dATP, and APAF-1 and through the caspase recruitment domain (CARD). Several pieces of evidence on the role of ceramides during caspase cascade using caspase inhibitors to prevent sphingolipid-induced apoptosis suggest ceramide formation is associated with the execution phase of apoptosis. Modulation of cellular ceramide levels could be a useful approach to modulate cell regulation and apoptosis.

It was previously reported that increases in ceramide concentrations can stimulate apoptosis. Ceramides are a class of sphingolipids comprising fatty acid derivatives of a sphingoid, e.g., sphingosine, base (see, e.g., Stedman's Medical Dictionary (Illustrated), 24th edition (J. V. Basmajian et al., eds.), Williams and Wilkins, Baltimore (1982), p. 99). Different ceramides are characterized by different fatty acids linked to the sphingoid base. For example, stearic acid can be attached to the amide group of sphingosine to give rise to the ceramide $CH_3(CH_2)_{12}CH=CH\text{-}CHOH\text{-}CH(CH_2OH)\text{-}NH\text{-}CO\text{-}(CH_2)_{16}CH_3$. Shorter- or longer-chain fatty acids can also be linked to the sphingoid base. Applicants have previously reported that attachment of certain chemical groups to sphingolipids and

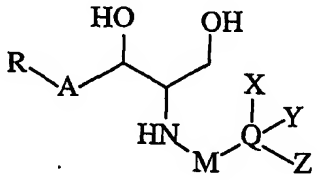
ceramides so as to form analogs of such compounds can inhibit bioconversion of ceramides to sphingomyelins, and can thereby lead to an apoptosis stimulating increase in ceramide concentrations.

Ceramides are found in all eukaryotic cell membranes, and are known to participate in a variety of critical cellular processes. Furthermore, certain sphingolipid compounds have been found to play a role in prevention of cellular proliferation. However, there has been no recognition in the prior art of the specific halogentated ceramides of the invention and their use in treating neoplastic diseases, metabolic conditions such as diabetes, inflammations and viral infections.

Short-chain (C_2-C_6), cell-permeable ceramides have been shown to directly affect endogenous ceramide levels, these short-chain ceramides mainly have been used for studying ceramide-mediated cellular functions. Further, though some reports have suggested that halogenated ceramides may be potentially useful as therapeutics for various pathologies of the nervous system, there has been no indication that halogenated ceramide derivatives would exhibit enhanced anti-neoplastic activity as compared to non-halogenated analogs, such as C2 and C6 ceramides.

Summary of the Invention

Briefly, the invention involves a compound of the formula I:



wherein:

R is C_nH_{2n+1}, where n is an integer of from 1-18;

A is CH_2-CH_2, CH_2-CH(OH), or cis, trans or cis+trans CH=CH;

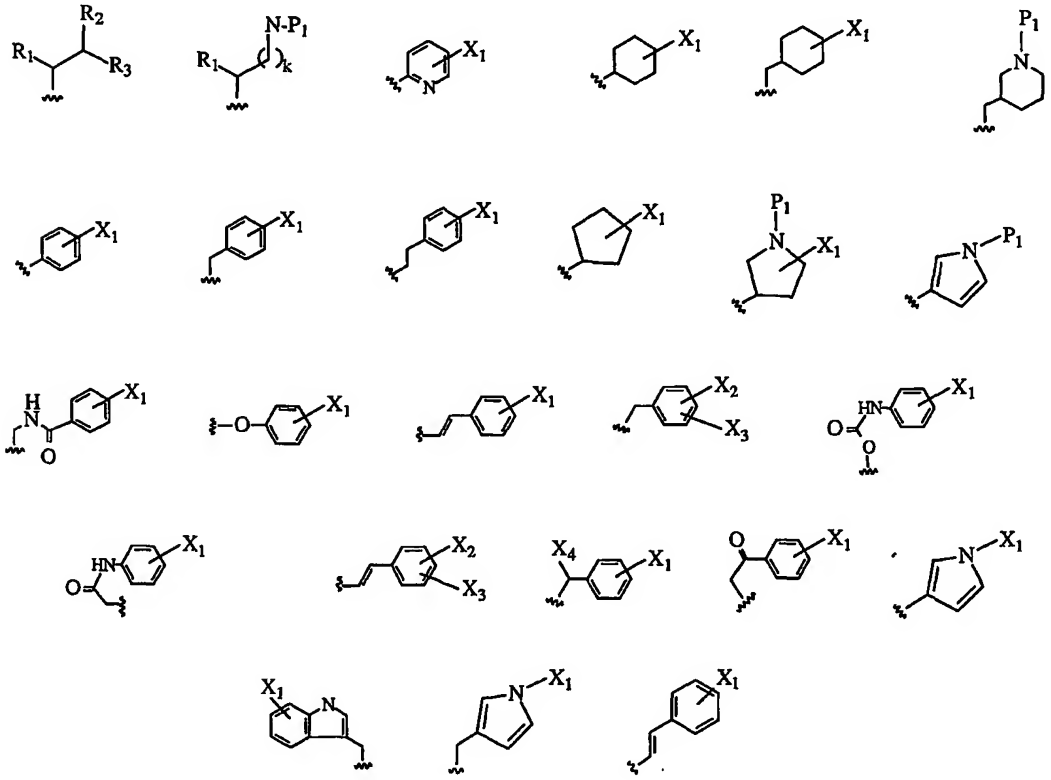
M is C(O) or CH_2;

Q is C, OC or $S(O_2)N$;

Y is H, OH, C_1-C_6 alkyl, C(O)OH, aryl, phenyl, NH_2, NO_2, C_6H_5, a halogen, NH-P_1, (O) or (S);

Z is H, C_6H_5, alkyl, NH_2, NH-P_1 or C(O)OH, wherein when Y is (O) or (S), Z is not present, and wherein when X, Y and Z are present as different moieties, the compound has an *R*, an *S*, or any combination of the *R* and *S* configurations about the α-carbon;

X is F, Cl, Br, I, C_6H_5, O-Si$(CH_3)_3$, O-Si$(C_4H_9)_3$, O-Si$(C_6H_5)_3$, C_1-C_{17} alkyl, -CH_2-O-X_1, or



k is integer from 0 to 6;

R_1, R_2, and R_3 are each independently H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 cycloalkyl, or aryl;

X_1 is H, OH, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, C_2-C_6 cycloalkyl, aryl, phenyl, a halogen, NO_2, CN, C(O)H, C(O)OH, $C(O)OCH_3$, $COCH_3$, CH_2OH, NH_2, N_3, $NHCH_3$, $CONH_2$, $N(CH_2CH_2)_2Cl_2$, $B(OH)_2$, furyl or aryl sulfonate;

X_2 is $-O-CH_2-$, and X_3 is $-O-CH_2-$ or $-O-$, such that X_2 and X_3 taken together form a heterocyclic ring; and

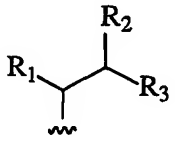
P_1 is H or an amino protecting group. The ceramide derivative can be one or any combination of the D-*erythro*, D-*threo*, L-*erythro* and L-*threo* configurations, as well as one or any combination of (+) and (-) optical isomers.

The following combinations of substituents are preferred for formula I:

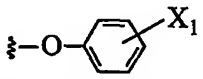
Each of Y and Z is independently H or C_6H_5.

M is C(O), X is Br or a C_1-C_{17} alkyl group, Q is C, Z is H, C_6H_5, an alkyl group or C(O)OH.

M is C(O), and X is



M is C(O), X is

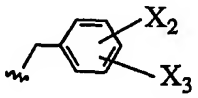


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, CH_3, NH_2, or NO_2; and

Z is H.

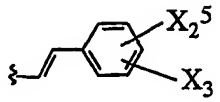
M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and

Z is H.

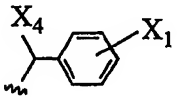
M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and

Z is H.

M is C(O), X is



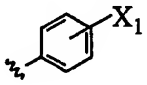
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

X_4 is H, a halogen, OH, OCH_3, NO_2, CN, C_1-C_6 alkyl or C_2-C_6 cycloalkyl;

Y is H, CH_3, NH_2, or NO_2; and

Z is H.

M is C(O), X is

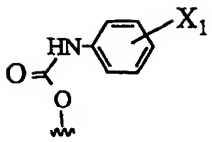


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

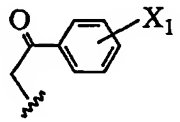
Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is



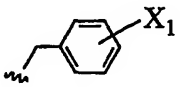
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H; and
Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is



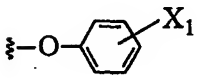
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H; and
Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is



X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

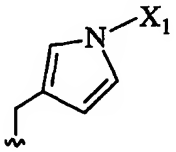
M is C(O), X is



X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

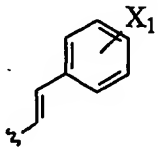
M is C(O); X is $-CH_2$-O-X_1;
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

M is C(O), X is



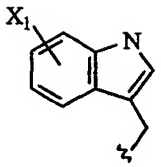
X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

M is C(O), X is



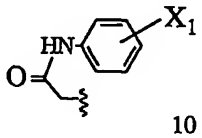
X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl; and
Z is NH_2 or NH-P_1.

M is C(O), X is



X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;
Y is H, OH, a halogen, NH_2, NH-P_1, (O) or (S).

M is C(O), X is



and X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl.

P_1 is Boc, Fmoc, Troc, silyl, sulfonyl, acetyl or benzyl.

The invention also encompasses a pharmaceutical composition comprising the ceramide derivative described above, and a liposome having a lipid bilayer that comprises a lipid and the ceramide derivative described above. The invention encompasses methods of treating an animal afflicted with cancer, an inflammatory disease or a viral infection by administering an anti-cancer, anti-inflammatory or anti-viral effective amount of the ceramide derivative to the animal.

Brief Description of the Drawings

Figs. 1A-1H are a series of fluorescence micrographs showing 2-Br C2 ceramide induced apoptosis in human tumor cell lines. Control (untreated) U937 (Fig. 1A), MCF7 (Fig. 1E), MCF7/ADR (Fig 1G) or 2-Br C2 ceramide treated cells: U937, with 2 μM for 2 hr (Fig 1B), 5 μM for 2 hr (Fig. 1C), 5 μM for 3 hr (Fig. 1D), MCF7, with 25 μM for 20 hr (Fig. 1F), MCF7/ADR, with 1 μM for 20 hr (Fig. 1H), were labeled *in situ* with biotin-dUTP and counterstained with

fluoresceinated avidin. Only a few cells were labeled in untreated samples and the unlabeled cells are barely visible. The photographs were taken using a fluorescence microscope with 40X objective lens.

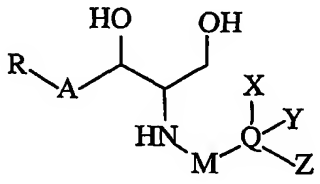
Figs. 2A-2D are a series of graphical depictions of 2-Br C2 ceramide induced apoptosis in U937 cells. For Fig. 2A, cells were treated with 0, 2, or 5 μM of 2-Br C2 ceramide for the indicated hours and then processed using TUNEL assay. The dUTP-biotin labeled (apoptotic) cells were distinguished from unlabeled ones and quantified using flow cytometry. Mean values ± SE from at least three independent experiments are shown in 5 μM treated samples. Histograms shown are from a representative experiment, in which cells were either untreated (Fig. 2B), treated with 100 μM C2 ceramide (Fig. 2C), or 5 μM 2-Br C2 ceramide (Fig. 2D) for 5 hr. Cells were double stained with Propidium Iodide for DNA content (X-axis) and Biotin-dUTP for apoptotic cells (Y-axis). R2 and R3 regions represent the non-apoptotic and apoptotic populations, respectively. Total event contains 15,000 cells and doublets were gated out of the statistic regions.

Figs. 3A and 3B are plots showing caspase inhibitors preventing U937 cells from undergoing 2-Br C2 ceramide induced apoptosis. For Fig. 3A, various peptide inhibitors were incubated with cells an hour prior to drug treatment. Cells were further treated with 2-Br C2 ceramide for additional 3 hrs and processed using TUNEL assay as described previously. Fig. 3B indicates ZIETD-FMK inhibition of 2-Br C2 ceramide induced apoptosis in a dose-dependent manner. Increasing doses of ZIETD-FMK were added to cells prior to 2-Br C2 ceramide treatment. Data shown are representative of two independent experiments.

Figs. 4A and 4B are graphs showing caspase activation by 2-Br C2 ceramide treatment. U937 cells were treated with 5 μM 2-Br C2 ceramide for indicated times were processed for cytosolic extract as described in materials and methods. 50 μg of total protein from the extracts were used in all samples and the volume was normalized with lysis buffer. IETDase (Fig 4A) and DEVDase (Fig. 4B) activity in extracts was measured with the fluorogenic substrate, Ac-IETD-AFC and Ac-DEVD-AFC, respectively.

Detailed Description of the Invention

The present invention comprises novel ceramide derivatives that show significant anti-neoplastic activity. The ceramide derivatives are of the formula I:



I

wherein:

R is C_nH_{2n+1}, where n is an integer of from 1-18;

A is CH_2-CH_2, CH_2-CH(OH), or cis, trans or cis+trans CH=CH;

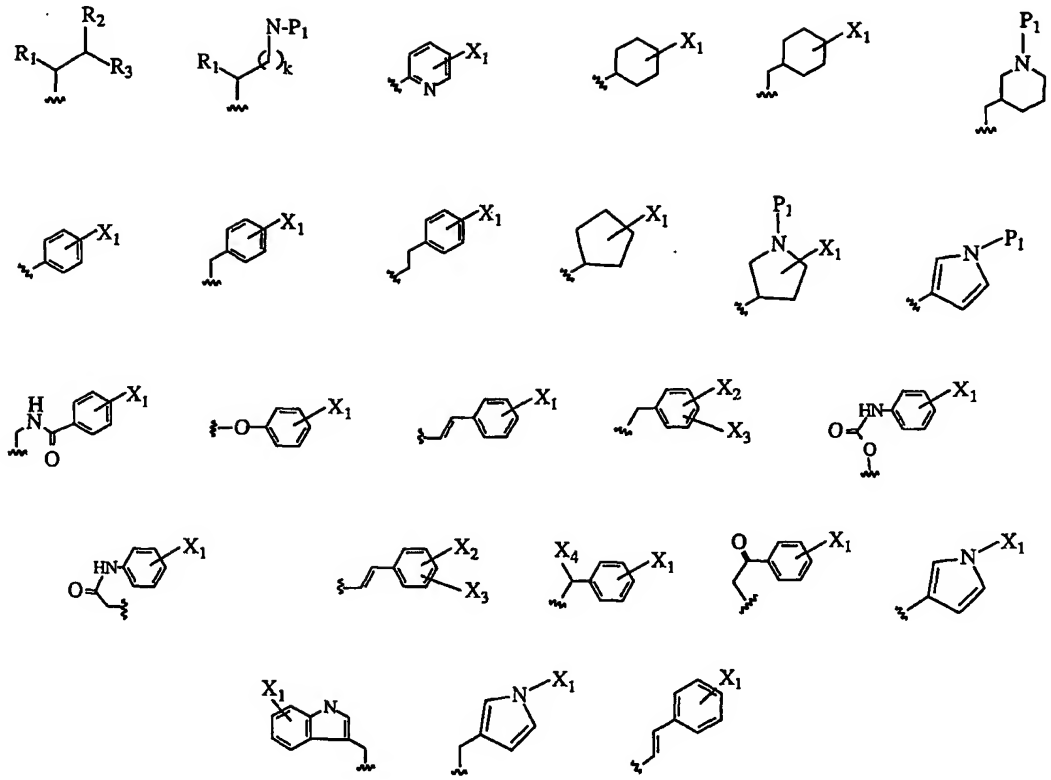
M is C(O) or CH_2;

Q is C, OC or $S(O_2)N$;

Y is H, OH, C_1-C_6 alkyl, C(O)OH, aryl, phenyl, NH_2, NO_2, C_6H_5, a halogen, NH-P_1, (O) or (S);

Z is H, C_6H_5, alkyl, NH_2, NH-P_1 or C(O)OH, wherein when Y is (O) or (S), Z is not present, and wherein when X, Y and Z are present as different moieties, the compound has an *R*, an *S*, or any combination of the *R* and *S* configurations about the α-carbon;

X is F, Cl, Br, I, C_6H_5, O-Si$(CH_3)_3$, O-Si$(C_4H_9)_3$, O-Si$(C_6H_5)_3$, C_1-C_{17} alkyl, -CH_2-O-X_1, or



k is an integer from 0 to 6;

R_1, R_2, and R_3 are each independently H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 cycloalkyl, or aryl;

X_1 is H, OH, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, C_2-C_6 cycloalkyl, aryl, phenyl, a halogen, NO_2, CN, C(O)H, C(O)OH, $C(O)OCH_3$, $COCH_3$, CH_2OH, NH_2, N_3, $NHCH_3$, $CONH_2$, $N(CH_2CH_2)_2Cl_2$, $B(OH)_2$, furyl or aryl sulfonate;

X_2 is $-O-CH_2-$, and X_3 is $-O-CH_2-$ or $-O-$, such that X_2 and X_3 taken together form a heterocyclic ring; and

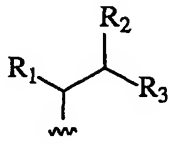
P_1 is H or an amino protecting group. The ceramide derivative can be one or any combination of the D-*erythro*, D-*threo*, L-*erythro* or L-*threo* confirmations, but is preferably D-*erythro*. Additionally, the ceramide derivatives can consist of a (+) optical isomer exclusively, a (-) optical isomer exclusively, or any combination of (+) and (-) optical isomers.

The following combinations of substituents are preferred for formula I:

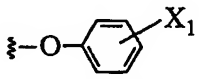
Each of Y and Z is independently H or C_6H_5.

M is C(O), X is Br or a C_1-C_{17} alkyl group, Q is C, Z is H, C_6H_5, an alkyl group or C(O)OH.

M is C(O), and X is

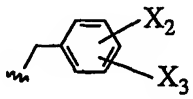


M is C(O), X is



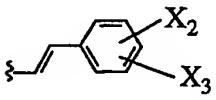
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;
Y is H, CH_3, NH_2, or NO_2; and
Z is H.

M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and
Z is H.

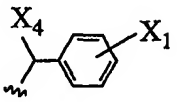
M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and

Z is H.

M is C(O), X is



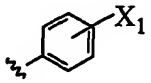
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

X_4 is H, a halogen, OH, OCH_3, NO_2, CN, C_1-C_6 alkyl or C_2-C_6 cycloalkyl;

Y is H, CH_3, NH_2, or NO_2; and

Z is H.

M is C(O), X is

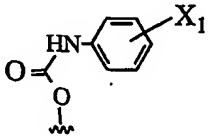


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is

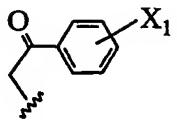


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is

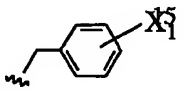


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

M is C(O), X is

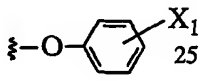


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

M is C(O), X is



X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

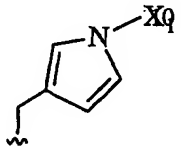
M is C(O); X is $-CH_2-O-X_1$;

X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

M is C(O), X is

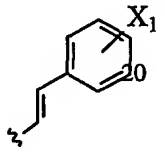


X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

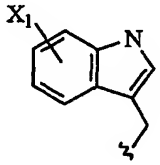
M is C(O), X is



X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl; and

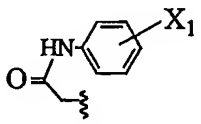
Z is NH_2 or NH-P_1.

M is C(O), X is



X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;
Y is H, OH, a halogen, NH_2, NH-P_1, (O) or (S).

M is C(O), X is



and X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl.

P_1 is Boc, Fmoc, Troc, silyl, sulfonyl, acetyl or benzyl.

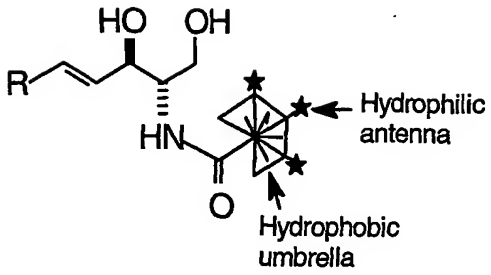
The invention also encompasses a pharmaceutical composition comprising the novel ceramide derivatives. The pharmaceutical composition may include a pharmaceutically acceptable carrier. The invention also comprises a method of treating an animal afflicted with cancer, an inflammatory disease or a viral infection by administering an anti-cancer, anti-inflammatory or anti-viral effective amount of the ceramide derivative to the animal. An "anti-cancer effective amount" is an amount that slows or stops the proliferation of cancer cells or other abnormally-proliferating cells, or causes cancer cell death. An "anti-inflammatory effective amount" is an amount that slows or stops an inflammatory response in a patient. An "anti-viral effective amount" is an amount that slows or stops the proliferation of a virus, or causes the death of a virus.

The methods used to synthesize the ceramide derivative are not particularly limited, and various techniques well known to those of ordinary skill in the art, may be used, such as those described in R. Selinger and Y. Lapidot, *J. Lipid Res.*, 7:174-175 (1966), incorporated in its entirety herein by reference. Illustrative examples of solution-phase syntheses follow, though solid-phase and combinatorial techniques may also be used. The examples are not intended to limit the scope of the invention defined in the appended claims.

Sphingosines and ceramides are formed in animal cells by the combination of palmitoyl CoA ($CH_3(CH_2)_{14}$-CO-S-CoA) and serine to give dehydrosphinganine ($CH_3(CH_2)_{14}$Co-$CH(NH_3)$- CH_2OH and CO_2 (see, e.g., L. Stryer, Biochemistry (2nd edition), W. H. Freeman and Co., New York, pp. 461-462). Dehydrosphinganine is converted to dihydrosphingosine ($CH_3(CH_2)_{14}$-CH(OH)-$CH(NH_3)$-CH_2OH) which is then converted to sphingosine ($CH_3(CH_2)_{12}CH$=CH-CH(OH)-$CH(NH_2)$-CH_2OH). A fatty acid is then linked to the amide group of sphingosine to give rise to a ceramide ($CH_3(CH_2)_{12}CH$=CH-CHOH-$CH(CH_2OH)$-NH-CO-R, where R is a fatty acid chain). A phosphorylcholine group ($PO_4CH_2CH_2$-$N(CH_3)_3$) can be attached to the ceramide at its hydroxyl group to produce a sphingomyelin ($CH_3(CH_2)_{12}CH$=CH-CHOH-$CH(CH_2PO_4CH_2CH_2$-$N(CH_3)_3$)-NH-CO-R). Sphingomyelinase can catalyze the hydrolytic removal of the phosphorylcholine from the sphingomyelin to give rise to a ceramide. Reverse hydrolysis of the ceramide can give rise to a sphingomyelin.

Without limiting ourselves by theory, the growth inhibitory data collected on the inventive ceramides and presented below suggest that there is a structure-activity relationship depending upon the nature of the X, Y, and Z groups attached. Since variations in potency are found with changes in short-chain residues (as is the case with 2-Bromo Cx, 2 or 3-Methyl Cx, 2 or 3-Phenyl Cx ceramide series), it is likely that hydrophobicity plays a key role in distinguishing the cellular targets. Those targets may have active sites that could accommodate only smaller size hydrophobic moieties. Having a hydrophilic moiety tethered to the hydrophobic moiety (as is the case with N-Tyrosine (4-*O*-^{t}Bu)¡ N-Boc-Phenylalanine (4-N,N-dichloroethyl), and N-Boc-Phenylalanine (4-CH_2NH-*i*Propyl ceramide compounds) may assist in enhancing cytotoxicity.

The following depiction suggests the theoretical concept by which target binding is increased in order to enhance biological activity.



The compounds of this invention, comprising alkyl chains of varying length, are synthesized by a number of routes well known to and readily practiced by ordinarily skilled artisans, given the teachings of this Invention (see, for example, below, wherein "rf" refers to one of the following references: 1: J. Am. Chem. Soc., 94: 6190 (1972); 2: J. Org. Chem. 59: 668 (1994); 3: Angew. Chem., Intl. Ed. (English), 17: 569 (1978); 4: Vogel's Textbook of Practical Organic Chemistry (5th ed.), pp. 769-780); 5: J. Org. Chem. 40: 574 (2975); 6: J. Org. Chem. 59: 182 (1994); 7: J. Org. Chem. 25: 2098 (1960); 8: Synthesis (1985): pp. 253-268; 9: J. Chem. Soc. (1953): p. 2548; 10: J. Am. Chem. Soc. 90: 4462, 4464 (1968); 11: Oxidations in Organic Chemistry (Am. Chem. Soc, Washington, D.C. (1990), pp. 60-64; 12: J. Med. Chem. 30 1326 (1987); 13: Synth. Commun. 9: 757 (1979); 14: The Chemistry of Amides (J. Wiley & Sons, New York (2970)), pp. 795-801; 15: J. Med. Chem. 37: 2896 (1994);4: J. Med. Chem. 30: 1326 (1987); 16: Rec. Chem. Prog. 29: 85 (1968); and 17: Phospholipids Handbook (Marcell Dekker, Inc., New York (1993), p. 97)). For example, such artisans would use a sphingosine or a ceramide as their starting material. Alkyl chains of varying length can be attached thereto, or removed therefrom, by known means.

Also provided herein is a pharmaceutical composition comprising the compound of this invention and a pharmaceutically acceptable carrier; the composition can also comprise an additional bioactive agent. "Pharmaceutically acceptable carriers" as used herein are generally intended for use in connection with the administration of lipids and liposomes, including liposomal bioactive agent formulations, to animals, including humans. Pharmaceutically acceptable carriers are generally formulated according to a number of factors well within the purview of the ordinarily skilled artisan to determine

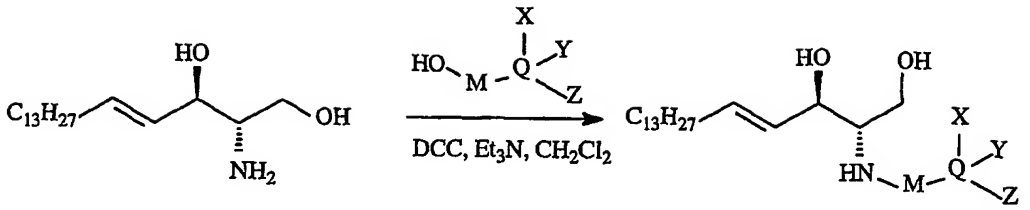
and account for, including without limitation: the particular liposomal bioactive agent used, its concentration, stability and intended bioavailability; the disease, disorder or condition being treated with the liposomal composition; the subject, its age, size and general condition; and the composition's intended route of administration, e.g., nasal, oral, ophthalmic, topical, transdermal, vaginal, subcutaneous, intramammary, intraperitoneal, intravenous, or intramuscular (see, for example, Nairn, J.G., *Pharmaceutical Sciences*, Mack Publishing Co. (1985)). Typical pharmaceutically acceptable carriers used in parenteral bioactive agent administration include, for example, D5W, an aqueous solution containing 5% weight by volume of dextrose, and physiological saline. Pharmaceutically acceptable carriers can contain additional ingredients, for example those which enhance the stability of the active ingredients included, such as preservatives and anti-oxidants.

Example 1

General Procedure for the Synthesis of Ceramide Derivatives

In this and the following examples, sphingosine (synthetic and swine brain), with purity >98%, was obtained from Avanti Polar Lipids (Alabaster, AL) or Matreya, Inc. (Pleasant Gap, PA). All other reagents used in the synthesis were obtained either from Aldrich (Milwaukee, WI) or from Fluka (Ronkonkoma, NY). Solvents used for the reactions and purification, unless stated otherwise, were obtained from Aldrich.

As generally shown in Scheme 1 below, sphingosine was reacted with the carboxylic anhydride or chloride and triethyl amine (Et_3N) in methylene chloride (CH_2Cl_2) at room temperature. The time of completion of most of the reactions was < 5 min. The reactions were monitored by thin layer chromatography (TLC) using ceramide (C_6, Sigma) as a standard. The R_f values for most of the derivatives were within 0.4-0.6 in $CHCl_3$/MeOH (90:10). After the reaction, if carboxylic acid was used, the white precipitate of dicyclohexylurea was filtered through a Celite pad. Solvent was evaporated under vacuo and the residue was purified by TLC in $CHCl_3$:MeOH (93:7) as the eluents.

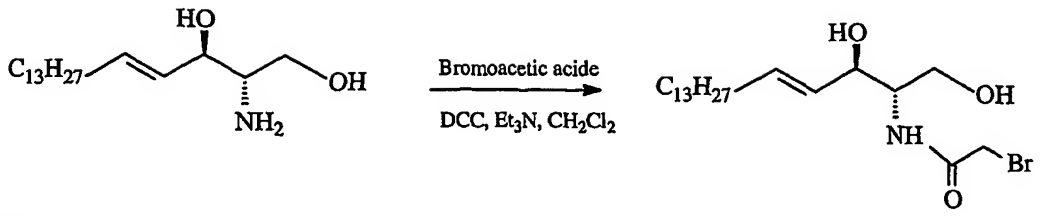


Scheme 1 showing the general route to the synthesis of ceramide derivatives.

Example 2

Procedure for the Synthesis of N-2-Bromoacetyl Sphingosine (2-Br C2 Ceramide)

As generally shown in Scheme 2 below, sphingosine (200 mg, 0.67 mmol, Avanti Polar Lipids) was added to 15 ml of anhydrous dichloromethane solution containing 2-bromoacetic acid (111 mg, 0.80 mmol; Aldrich Chemicals) and 1,3-dicyclohexylcarbodiimide (165 mg, 0.8 mmol, Aldrich Chemicals). The reaction mixture was brought to 0° C with the aid of an ice-water bath and then triethylamine (73 mg, 100 µl, 1 mmol, Fluka) was added. After 30-min reaction at 0° C, the white precipitate of dicyclohexyl urea was filtered through a Celite bed, and the filtrate was concentrated under vacuo. The residue obtained was purified by preparative TLC (silica gel, 2000 micron plate, Analtech, Newark, DE) using chloroform:methanol (9:1) to yield the desired product with R_f = 0.5 (9:1, chloroform:methanol). Finally, the product was lyophilized from cyclohexane to yield 80 mg of white flaky powder, which was characterized by ^{1}H NMR and mass spectrometry (MS). The characteristic proton signals, underlined, were at δ (ppm in $CDCl_3$): 7.2 (d, 1H, N<u>H</u>), 5.8 (m, 1H, C<u>H</u>=CH), 5.5 (dd, 1H, CH=C<u>H</u>), 4.3 (s, 1H, C<u>H</u>-OH), 4.0 (dd, 2H, C<u>H$_2$</u>OH), 3.9 (s, 2H, C<u>H$_2$</u>Br), 3.7 (m, 1H, C<u>H</u>-NH), 2.5 (bs, 1H, OH), 2.4 (s, 1H, OH), 2.1 (m, 2H, allylic C<u>H$_2$</u>), 1.6 (s, 2H, homoallylic C<u>H$_2$</u>), 1.3 (s, 20H, (C<u>H$_2$</u>)$_{10}$), 0.9 (app. t, 3H, □-C<u>H$_3$</u>). ESI-MS: m/z 420 ($M.H^+$), parent m/z 402 ($M-H_2O).H^+$.



Scheme 2

Other ceramides varying in acyl chain length from C_4 to C_{20} were prepared by the same procedure as described above except the corresponding 2-bromo carboxylic acids were used. The resulting products were characterized by TLC and 1H NMR. 2-chloro and 2-iodo C_2 ceramides were prepared by the same procedure as described above except 2-chloro acetic acid and 2-iodo acetic acid were used, respectively, and characterized by TLC and ^1H NMR.

Example 3

Anti-neoplastic activity evaluations

Various ceramides were prepared as described above and evaluated for anti-neoplastic activity against several established tumor cell lines using Sulforhodamine B (SRB) assays (described below). The anti-neoplastic activity of the ceramides against particular leukemia lines was also evaluated as described below. HT29, human colon carcinoma, was obtained from the American type culture collection (Rockville, MD) and the following cell lines were obtained from DCT Tumor Repository (Frederick, MD): MCF7 human breast tumor, MCF7/ADR (MCF7 adriamycin resistant subline), A549 human non small cell lung cancer, P388 murine leukemia, P388/ADR (P388 adriamycin resistant subline), and U937 human promyelocytic leukemia. All lines were grown in Complete Medium (RPMI 1640 medium containing 10% fetal bovine serum (GIBCO)) in an atmosphere of 5% CO_2 at 37 ºC. Depending on the cell type, 3,000 to 10,000 adherent cells were plated onto 96-well plates in a volume of 100 µl per well one day prior to the ceramide incubation. Ceramides were first dissolved in DMSO (dimethyl sulfoxide) at a stock concentration of 20 mM, which is 400 times the desired final maximum test

concentration. The stock solutions were then diluted with complete medium to twice the desired final concentration. 100 µl aliquots of each dilution were added to the designated wells. After 3 days incubation, cell growth was determined by SRB assay as described in Example 4. For some apoptosis studies, 20 µM protease inhibitors (all purchased from Enzyme System Products, CA), Cbz-Val-Asp(O-methyl)-fluoromethyl ketone (ZVAD-FMK), Cbz-Asp-Glu-Val-Asp(O-methyl)-fluoromethyl ketone (ZDEVD-FMK), Cbz-Ile-Glu-Thr-Asp(O-methyl)-fluoromethyl ketone (ZIETD-FMK), Cbz-Leu-Glu-His-Asp(O-methyl)-fluoromethyl ketone (ZLEHD-FMK), or Cbz-Phe-Ala(O-methyl)-fluoromethyl ketone (ZFA-FMK), were incubated with cells one hour prior to ceramide incubation (Cbz = the protecting group benzylcarbomethoxy).

Example 4

Cell Growth Assay I

Sulforhodamine B (SRB) assays were performed as described by Monk, A. *et al.*, *J Natl Cancer Inst*, 83:757-766 (1991), (incorporated herein by reference), with minor modifications. Following incubation with experimental ceramide derivatives or control compounds (i.e., without drug), cells were fixed with 50 µl of cold 50% (wt/vol) trichloroacetic acid (TCA) for 60 minutes at 4 ºC. The supernatant was discarded, and the plates were washed six times with deionized water and air dried. The precipitate was stained with 100 µl SRB solution (0.4% wt/vol in 1% acetic acid) for 10 minutes at room temperature, and free SRB was removed by washing three times with 1% acetic acid, and the plates were air dried. Bound SRB was solubilized with Tris buffer (10 mM), and the optical densities (ODs) were read using an automated plate reader (Bio-Rad, Model 3550-UV) at 490 nm. Background values were subtracted from the test data, and the data were calculated as % of control. The GI_{50} represents the concentration of test agent resulting in 50% of net growth compared to that of the control untreated samples. In this assay, ODs were also taken at time 0 (the time when drugs were added) from an extra plate. If the ODs of the tested sample were less than that the time 0 sample, cell death had occurred. Percentage growth was calculated as described by Peters, A. C. *et al.*, *Lipids*, 32:1045-1054, 1997 (incorporated herein by reference). Briefly, percentage growth was calculated

as follows: $(T - T_0)/(C - T_0) \times 100$, where T = mean optical density of treated wells at a given drug concentration, T_0 = mean optical density of time zero wells, and C = mean optical density of control wells. If $T < T_0$, which indicates that cell death has occurred, then percentage cell death was calculated as $(T - T_0)/(T_0) \times 100$.

Example 5

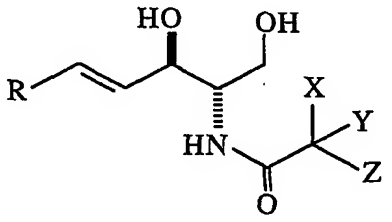
Cell growth assay II

To determine the GI_{50} values in the leukemia lines (suspension cells), cell numbers were directly counted instead of using the SRB assay which determines total cell protein. One day prior to drug treatment, 40,000/ cell wells were seeded into 24-well plates in a volume of 0.5 ml. Stock solutions were diluted with complete medium to twice the desired final concentrations, and then 0.5 ml aliquots of each dilution were added to the designated wells. After 3 days incubation, cell growth was determined by counting cell number using a Coulter counter (Z-M, Coulter). Cell counts were also taken at time 0 and subtracted from the test results to give net growth. The GI_{50} represents the concentration of test agent resulting in 50% of net growth compared to that of the untreated control samples.

Various ceramides prepared as described above were evaluated using the materials and techniques described above. The evaluations are detailed in the examples below.

Example 6

Ceramides of the following formula were prepared as described above:



with $R=(CH_2)_{12}CH_3$, Z=H, and X and Y as defined in Tables 1 and 2 below. Thus, when both X and Y are H, the structure of the C_2 ceramide is depicted. The GI_{50} values were

determined by the SRB assay as described above. Cells were treated with the ceramide derivatives as indicated for 3 days and then fixed for the assay. The results are reported in Tables 1 and 2.

Table 1. The GI_{50} of Various Ceramides in Human tumor lines

Ceramide	Cell Death at 50 μM	GI_{50} (μM) HT-29	GI_{50} (μM) A-549	GI_{50} (μM) MCF-7	GI_{50} (μM) MCF-7/ADR
X,Y=H	+	20.0*	31.7	36.6	29.0*
X=H; Y=$(CH_2)_3CH_3$	+	4.1	4.6	12.0	12.3
X=Br; Y=H	+	3.2	5.2*	5.6*	1.2
X=Br; Y=CH_3	+	3.6	5.4	8.1	6.9
X=Br; Y=CH_2CH_3	+	5.5	6.3	6.3	4.8
X=Br; Y=$(CH_2)_3CH_3$	+	3.3	4.2	4.7	5.3
X=Br; Y=$(CH_2)_5CH_3$	+	nd	15.5	23.1	20.2
X=Br; Y=$(CH_2)_7CH_3$	-	nd	< 50	< 50	nd
X=Br; Y=$(CH_2)_9CH_3$	-	nd	> 50	> 50	nd
X=Br; Y=$(CH_2)_{13}CH_3$	-	nd	> 50	> 50	nd
X=Cl; Y=H	+	14.8	16.8	16.8	11.9
X=I; Y=H	+	7.1	7.8	8.3	6.0

Table 2. The GI_{50} (μM) of 2-Br C2 ceramide and C2 ceramide in Leukemia cell lines

Cell lines	X=Br; Y=H	X,Y=H
P388	0.2 ± 0.01	30.6*
P388/ADR	0.6 ± 0.06	24.3*
U937	1.0 ± 0.2	22.0 ± 0.9

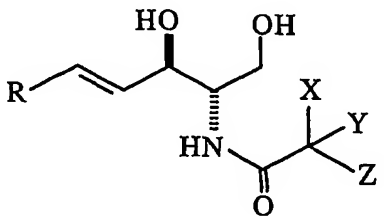
Unless otherwise marked, the results in Tables 1 and 2 were the average of three samples of duplicates (total of six data points) from a single experiment. Those marked by * were the average of two independent experiments (total of twelve data points), while results presented as mean ± S.E. were from three or more independent experiments. Numbers containing > or < were an estimated values based on % control in the prescreening test rather than from a full scale GI_{50} study. nd: not determined. (+): Cell death observed (when the OD of the test samples is less than the time 0 sample) at 50 μM, the maximum test concentration. (-): Cell death not observed.

Although the only difference between 2-Br C2 ceramide and C2 ceramide (a cell-permeable synthetic ceramide) is the replacement of the hydrogen with bromine at the 2-position of ceramide, this modification greatly enhanced growth inhibition compared to the parent compound. We compared the GI_{50}'s of the two ceramides in several murine and human cancer cell lines and surprisingly found that 2-Br C2 ceramide is 5 to 50 times more potent compared to C2 parent ceramide. While the GI_{50}'s for C2 ceramide ranged from 20 to 30 μM, the GI_{50}'s for 2-Br C2 ceramide were from 0.2-6 μM. Interestingly, 2-Br-C2 ceramide was more potent in leukemia (< 1 μM) than in solid tumor lines (1- 6 μM) and also active in drug resistant lines (MCF7/ADR vs. MCF7 and P388 vs. P388/ADR). While not limiting ourselves by theory, we believe that the enhanced potency of 2-Br-C2 ceramide compared to C2 ceramide could result from changes in cell permeation, compartmentalization and cell metabolism, due to the altered structure and conformation of the ceramide. The 2-Cl- and 2-I- C2 ceramides showed less activity than the 2-Br C2 ceramide against tumor cell growth, though the 2-I was surprisingly much better than the 2-Cl.

As in the C2 ceramides, the 2-Br-C6 ceramide surprisingly showed more potency than the C6 ceramide, though the differences were not as dramatic as those between the 2-Br-C2 and C2 ceramides. Potency decreased in the 2-Br-C8 ceramide, and the 2-Br-C10 to C16 ceramides were relatively inactive in these *in vitro* tests.

Example 7

The effect of length of the hydrocarbon chain in the sphingoid base on 2-Br-C2 ceramides anti-neoplastic activity was investigated by preparing, as described above, ceramides of the following formula:



with X=Br, Y=H, Z=H, and R as defined in Table 3 below. The GI_{50} values were determined by the SRB assay as described above. Cells were treated with ceramide derivatives as indicated for 3 days and then fixed for the assay. The results are reported in Table 3.

Table 3. The GI_{50} (μM) of 2-BrC2 ceramide-derivatives with various length of hydrocarbon chain in the sphingoid base

R	Cell Death at 50 μM	GI_{50} (μM) HT-29	GI_{50} (μM) A-549	GI_{50} (μM) MCF-7	GI_{50} (μM) MCF-7/ADR
$(CH_2)_4CH_3$	+	9.9	7.1	1.2	3.0
$(CH_2)_6CH_3$	+	7.2	7.5	3.7	1.8
$(CH_2)_8CH_3$	+	13.8	13.2	7.4	3.0
$(CH_2)_{10}CH_3$	+	17.4	24.4	12.3	6.3
$(CH_2)_{12}CH_3$	+	3.7	5.0	5.9	0.9
$(CH_2)_{14}CH_3$	+	6.3	6.1	5.4	< 1

Numbers in Table 3 containing > or < are based on the highest or the lowest test concentration in a full scale GI_{50} study. (+): Cell death observed (when the OD of the test samples is less than the time 0 sample) at 50 μM, the maximum test concentration.

Though all of the 2-Br-C2 ceramide derivatives showed anti-neoplastic activity, there was a decrease in activity for the C14 and C16 (sphingoid base hydrocarbon chain) ceramides. The C10, C12 and C20 (sphingoid base hydrocarbon chain) ceramides surprisingly showed greater potency against the MCF-7 cell line than the "natural" (C18) ceramide.

Example 8

The activity of the L-threo and D-erythro conformational isomers of 2-Br-C2 ceramide (N-2-Bromoacetyl sphingosine) against human tumor cells was investigated. GI_{50} values of the L-threo and D-erythro isomers (prepared as described above) were determined by the SRB assay as described above, and the results are reported in Table 4.

Table 4. The GI_{50} of ceramide conformational-isomers in human tumor cells

Compound (Ceramide)	Cell Death at 50 μM	GI_{50} (μM) HT-29	GI_{50} (μM) A-549	GI_{50} (μM) MCF-7	GI_{50} (μM) MCF-7/ADR
L-threo 2-BrC2-	+	6.4	10.9	5.5	< 1
D-erythro 2-BrC2-	+	3.7	5.0	5.9	0.9

The Table 4 results were the average of three samples of duplicates (total of six data points) from a single experiment. Numbers containing > or < were an estimated values based on % control in the prescreening test rather than from a full scale GI_{50} study. (+) Cell death observed (when the OD of the test samples is less than the time 0 sample) at 50 μM, the maximum test concentration.

As Table 4 indicates, the D-erythro isomer showed higher anti-tumor activity against the HT-29 and A-549 cell lines than the L-threo isomer. Both isomers were similarly effective against the MCF-7 and MCF-7/ADR lines.

Example 9

TUNEL Assay

To determine whether 2-Br C2 ceramide induced apoptosis, TUNEL (Terminal transferase mediated dUTP nick end labeling) assays were performed as described Gorczyca et al., *Cancer Res.*, *53*: 1945-1951 (1993). Briefly, following drug treatment, 2 x 10^6 cells were incubated with 1% formaldehyde in PBS (pH 7.4) for 20 min at room temperature and then fixed with 70% ice- cold ethanol at -20 °C for 1 to 3 days. Samples were rehydrated with PBS (pH 7.4) before suspension in 50 μl of a solution containing 1x TdT buffer (25 mM Tris-HCl, 200 mM Potassium cacodylate), 2.5 mM cobalt chloride, 0.5 n mole Biotin-16-dUTP, and 10 unit Terminal transferase (all above chemicals obtained from Roche Molecular Biochemicals) at 37 °C for 30 mins. Samples were then washed with cold PBS and resuspended in 100 μl of a staining solution (fluoresceinated avidin (1:150), 4x saline sodium citrate buffer, 0.1% Triton X-100, and 5% nonfat dry milk) for 30 mins at room temperature in the dark. For flow cytometric studies, cells were double stained with propidium Iodide (10 μg/ml) in PBS with 0.1% RNAse overnight at 4 °C. To observe samples using fluorescence microscopy, adherent cells were plated onto chamber slides (Falcon, NJ) before drug treatment and suspension cells were spun down to slides using a Cytospin (800 rpm, 4 mins) after drug treatment. The reaction volume was adjusted to cover the slides, and mounting fluid (Vector, CA) was applied to the slides after assay. Photographs were taken using a fluorescence microscope with a 40X lens, and are shown in Fig. 1 ((A) control (untreated) U937; (B) U937 treated with 2 μM 2-Br C2 ceramide for 2 hr; (C) U937 treated with 5 μM 2-Br C2 ceramide for 2 hr; (D) U937 treated with 5 μM 2-Br C2 ceramide for 3 hr; (E) control (untreated)

MCF7; (F) MCF7 treated with 25 μM 2-Br C2 ceramide for 20 hr; (G) control (untreated) MCF7/ADR; (H) MCF7/ADR treated with 1 μM 2-Br C2 ceramide for 20 hr).

The results shown in Fig. 1 reveal that 2- Br C2 ceramide induces apoptosis in U937 cells and in other solid tumor lines. The apoptotic (brighter) cells and condensed chromatin were observed in U937 cells as early as 2 hr following 2 μM 2-Br C2 ceramide treatment (Fig. 1B), and the number of apoptotic cells increased in a dose- and time- dependent manner (Fig.1B-D). At an early stage of apoptosis, chromatin granules were present within the nuclear membrane, however, by 3 hr of drug treatment, many cells seemed to have lost their integrity and the nuclei had disintegrated. Apoptosis was also observed in MCF7/ADR cells treated with the GI_{50} concentration (1 μM) of 2-Br C2 ceramide for 20 hr (Fig. 1H). In contrast to MCF7/ADR, the parent MCF7 cell line required a much higher drug concentration (4 X GI_{50}) for apoptosis induction (Fig. 1F) and we only observed positive staining of whole nuclei rather than condensed chromatin granules as observed in the other lines. In addition, with longer treatment, these cells became fragile and we found increased cell loss during assays.

In order to quantify 2-Br C2 ceramide-induced apoptosis, the same type of TUNEL assays was performed, and the results were quantified by flow cytometry. U937 cells were treated with 2 or 5 μM of 2-Br C2 ceramide for up to 24 hrs and % of apoptotic population was determined (Fig. 2A). Treatment with 2 μM (2 times GI_{50} concentration) 2-Br C2 ceramide induced a marginal increase of apoptotic cells (5- 8% above control) in 24 hr. However, with 5 μM treatment, this induction of apoptosis was detected by 2 hr after initiation of treatment where there were more than 10% cells undergoing apoptosis and this number increased to over 60% at 6 hr. Based on the results in Fig. 2A, cells were treated with either 100 μM of C2 ceramide (representative histogram shown in Fig. 2C) or 5 μM of 2-Br C2 ceramide (representative histogram shown in Fig. 2D) for 5 hrs and apoptosis studies were then performed and compared to the untreated control (representative histogram shown in Fig. 2B). These cells were double stained with Propidium Iodide for DNA content (x-axis of Figs. 2B-C) and Biotin-dUTP for apoptotic cells (y-axis of Figs. 2B-C). Although both C2 and 2-Br C2 ceramides induced apoptosis

in all phases of cell cycle, 2-Br C2 ceramide was at least three times more efficient in inducing apoptosis (12% vs. 43%) and at a far lower concentration (Fig. 2C, D).

Example 10

Signaling pathway of apoptosis activated by 2-Br C2 ceramide

To explore which signaling pathway of apoptosis was activated by 2-Br C2 ceramide, several cell permeable caspase inhibitors were added to U937 cells 1 hr prior to drug treatment at a final concentration of 20 μM. These inhibitors were previously shown to inhibit programmed cell death induced by various apoptogenic agents (Thornberry, *Chem Biol*, *5*: R97-103 (1998)). We used several inhibitors, ZVAD- FMK: a general inhibitor, ZDEVD- FMK: a inhibitor for Group II caspases and, to a lesser extent 8, and ZIETD- FMK: a specific inhibitor for caspase 8, ZLEHD-FMK: a specific inhibitor for caspase 9, and ZFA- FMK: a negative control, and studied their effects on 2-Br C2 induced apoptosis using the TUNEL assay. Because of the possible degradation of these peptide inhibitors, we only treated U937 cells with ceramides for additional 3 hrs after addition of the inhibitors before the harvest of cells for TUNEL assay. We found while ZVAD-, ZDEVD-, and ZIETD- FMK prevented 2-Br C2 ceramide-induced apoptosis, neither ZLEHD-FMK or the negative control (ZFA- FMK) affected (actually slightly enhanced) this apoptosis (Fig. 3A). A single agent, ZIETD-FMK which inhibits caspase 8 specifically, caused a dose-dependent inhibition of the apoptosis (Fig. 3B). More than 90% of 2-Br C2 ceramide-induced apoptosis was inhibited at as low as 5 μM ZIETD-FMK. These data suggest that the induction of apoptosis by 2-Br C2 ceramide is mediated through the caspase 8 rather than the caspase 9 cascade and it acts earlier in the pathway. In contrast to a previous report (Sweeney et al., *FEBS Lett*, *425*:61-65 (1998)) that ZIETD-FMK had almost no inhibitory effects on C2- or C6- ceramide-induced apoptosis in HL60 cells, we found that 20 μM of ZIETD-FMK was sufficient to inhibit 90% of the apoptosis induced by C2 ceramide in U937 cells (data not shown). Our data suggest that ceramide analogs can act not only upstream of the effector caspases but also upstream of the initiator caspase.

Example 11

Caspase activation in 2-Br C2 ceramide-treated cytosolic extract.

To investigate the specificity of the caspase activity induced by 2-Br C2 ceramide, we used the caspase specific substrates, IETD- and DEVD-AFC. Cytosolic extracts were prepared cell cytosolic extracts were prepared according to Tamm et al., *Cancer Res*, *58*:5315-5320 (1998), and reactions were carried out in 96 well plates as described by Cuvillier et al., *J Biol Chem*, *273*:2910-2916 (1998). 100 µl of reaction buffer (2X) containing 100 µM of Z-DEVE-AFC or Z-IETD-AFC (Enzyme System Products, CA), 20 mM Hepes (pH 7.4), 200 mM NaCl, 1 mM EDTA, 0.2% CHAPS, 10 mM DTT, and 20% Sucrose was add to an equal volume of buffer A (20 mM Hepes, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, and 1 mM DTT) containing 50 µg total protein. Enzymatic hydrolysis of substrates was measured by release of AFC (amino-trifluoromethyl coumarin) induring a 30-min period using a Cytofluor 4000 multi plate reader (PerSeptive Biosystems, MA) (excitation at 395 nm and emission at 490 nm). Caspase activity was measure as arbitrary fluorescence units and converted to fold increase over basal level in untreated (control) cells. Background fluorescence of substrate alone was subtracted in each coordinated sample. The accumulation of IETD- and DEVD-specific protease activity from untreated and treated U937 cells was determined (Fig. 4A & B). As can be seen, the activation of IETDase activity preceded that of DEVDase, although the latter had a greater and more sustained increase. This is in agreement with the concept that caspase 8 acts upstream in the signaling pathway and its activation occurs prior to the activation of effector caspases. This drug induced- activation of caspase 8 is a "transit" response; it peaked at 2 hr (2-fold), dropped below basal level right after and then gradually return to basal level (Fig. 4A). DEVDase activity reflects the activity of the down stream effectors, caspases 3, 6, and 7. The increase in DEVDase activity slowly followed that of IETDase, peaked at 3 hr, and remained elevated up to 6 hr (Fig. 4B), implying the down stream effectors remained active through the apoptosis process. These data further support our finding on the association of caspase 8 activation on 2-Br C2 ceramide-induced apoptosis. Scaffidi, et al., *J Biol Chem*, *274*:22532-22538 (1999) recent reported that only type II (eg. CEM and Jurkat), but not type I (eg. SKW6.4 and

H9) cells, are sensitive to C2 ceramide-induced apoptosis. Type I and type II cells of CD95 pathway differ in the kinetics and the levels of caspase 8 activation and in whether or not this activation bypasses mitochondria. Their data suggested that, in type II cells, C2 ceramide acts at the level of mitochondria, which is upstream of caspase 8 in the apoptosis pathway. In agreement with their finding, we also found that ceramide could act upstream of caspase 8 in U937 cells.

Example 12

Anti-proliferative testing of Peptidomimetic and Non-peptidomimetic Ceramides

Peptidomimetic ceramides (peptidomimetic = containing one or more an amino acid groups) and non-peptidometic ceramides were synthesized using the combinatorial parallel synthesis-technique described in Example 2. The ceramides obtained were then tested for anti-proliferative activity, and the results are reported in Tables 7 and 8 below. The GI_{50} values were determined by the SRB assay as described above. A "+" indicates that cell death was observed (when the OD of the test samples is less than the time 0 sample) at 50 μM, the maximum test concentration. "-" indicates that cell death was not observed at 50 μM.

Table 7: Ceramides analogs and their in vitro cytotoxicity properties

Non-Peptidomimetic Ceramide	HT29 (μM)	A549 (μM)	MCF-7 (μM)	MCF-7/ ADR (μM)
(*R*)-2-Phenylpropanoyl	6.2	9.7	11.7	18.9
(*S*)-2-Phenylpropanoyl	6.6	6.1	6.5	8.9
(*R*)-3-Phenylbutanoyl	3.2	3.4	3.9	5.4
(*S*)-2-Phenylbutanoyl	7.9	11.5	12.2	17.3
3-Methylbutanoyl (C4)	2.4	3.8	4.5	10.6
2-Methylcyclopropanoyl	2.8	5.7	6.3	10.5
(±)-2-Methylbutanoyl	3.5	3.9	6.2	3.8
2-Chloroacetyl (C2)	14.8	16.8	16.8	11.9
2-Bromoacetyl (C2)	5.2	3.3	6.8	0.8
2-Iodoacetyl (C2)	7.1	7.8	8.3	6.0
L-*threo*-2-Bromoacetyl (C2)	6.4	10.9	5.5	<1
2-Bromoacetyl (C2)-C10	10.9	7.6	3.6	3.2

2-Bromoacetyl (C2)-C12	16.8	11.9	7.7	2.7
2-Bromoacetyl (C2)-C14	>20	>20	>20	3.1
2-Bromoacetyl (C2)-C16	>20	>20	>20	7.6
2-Bromoacetyl (C2)-C20	3.0	2.0	7.0	0.4
(*S*)-2-Br-Propanoyl	4.2	12.6	3.8	7.8
Propanoyl (C-3)	3.0	10.2	12.2	12.68
3,3-di-Phenylpropanoyl	2.9	10.2	10.2	13.6
Butanoyl (C4)	2.5	7.2	13.4	18.7
(*S*)-2 Methylbutanoyl (C4)	1.5	5.2	4.7	5.2
(*S*)-2-Methylbutanoyl (C4) C-12	12.7	21.8	36.8	50.0
(*S*)-2-Methylbutanoyl (C4) C-14	5.3	7.9	9.8	17.5
(*S*)-2-Methylbutanoyl (C4) C-16	2.2	3.7	4.4	4.9
(*S*)-2-Methylbutanoyl (C4) C-20	3.5	3.2	3.9	4.8
R-3-Phenylbutanoyl (C4)	<2	2.5	3.6	5.4
2-Methylpentanoyl (C5)	2.4	3.4	5.3	6.2
3-Methylpentanoyl (C5)	1.6	1.4	5.9	11.7
4-Methylpentanoyl (C5)	0.9	10.0	9.9	13.8
3-Methylbutanoyl (C4)	1.1	2	3.8	6.8
3-(Trimethylsilyl)propionic	2.6	3.3	4.6	<1
3,4-(Methylenedioxyphenyl)-acetyl	6.2	10.8	12.6	>20/17
α-Phenylcyclopentanoyl	8.7	12.8	12.5	>20
2-Methoxyphenylpropanoyl	2.5	3.3	4.2	5.0
2-(4-Nitophenyl)-propanoyl	3.2	6.6	6.7	7.3
(Indole-3-acetyl)	5.6	7.4	7.2	10.9
(2-Norbornane carboxyl)	3.6	3.9	14.7	20.2
(Tosyl-3-pyrrolcarboxyl)	3.4	5.0	7.4	>10
3,3,5-Trimethylhexanoic	2.9	2.4	4.2	8.3
3-Methylpropanoyl (C3)	1.5	2.9	4.0	5.3
2-Ethylhexanoic	3.3	4.2	7.6	7.7
trans-2 methyl-2-pentenoic	3.1	6.1	11.2	12.6
Peptidomimetic Ceramide	**HT29 (μM)**	**A549 (μM)**	**MCF-7 (μM)**	**MCF-7/ ADR (μM)**
BOC-p-fluorophenylalanine	3.3	3.6	4.9	6.9
BOC-p-methylphenylalanine	3.1	3.6	5.6	7.2
BOC-p-chlorophenylalanine	3.9	5.1	6.2	7.1
BOC-p-cyanophenylalanine	1.1	4.2	5.6	6.7

NH_2-D-Homophenylalanine	4.5	11.0	6.7	>20
Boc-Homophenylalanine	1.9	3.0	4.0	6.7
Boc-Phenylalanine (4-N,N-dichloroethyl)	<2	4.5	4.0	5.6
Fmoc-Phenylalanine (4-I)	7.1	8.7	15	18
Fmoc-Norarganine (BOC)2	3.1	7.7	10.3	10.3
NH_2-Phenylalanine (4-COO-*t*Butyl)	6.7	6.1	4.2	9.3
4-*t*-Butylcyclohexanecar-boxylic (c/t)	6.0	8.2	11.4	14.9
Fmoc-Phenylalanine (4-CH_2N-isopropyl)	5.3	5.6	2.4	10.4
NH_2-Phenylalanine (4CH_2N-isopropanoyl)	2.8	3.0	2.0	>20
BOC-Phenylalanine [4-NH-(4-Bromobenzenesulfonyl)]	11.8	13.8	6.8	<2
N-Boc-Phenylalanine (4-Nitro-tetrahydrofuryol)	<2	8.1	8.1	7.3
Boc-cyclohexylalanine	3.5	4.3	7.1	11.2
Boc-D-cyclohexylalanine	3.3	3.6	8.1	16.5
NH_2-L(c/t)-Cyclohexylalanine-(4NH-Boc)	3.4	4.2	2.5	10.3
NH_2-o-Tyrosine (O-*t*Butyl)	1.8	1.9	1.1	11.2
NH_2-m-Tyrosine (O-*t*Butyl)	1.9	3.1	2.5	5.4
NH_2-p-Tyrosine (O-*t*Butyl)	0.8	2.4	3.3	4.2
BOC-Tyrosine (3,5-I_2)	3.4	9.4	11	13.6
NH_2-Tyrosine	4.6	5.6	5.4	>10
NH_2-Tyrosine (-O-Boc)	5.1	4.1	4.0	5.9
NH_2-D-Tyrosine (O-*t*Butyl)	4.4	6.0	6.4	13.6
NH_2-Homotyrosine	7.0	6.9	6.1	14.6
BOC-Norvalanine	2.5	4.2	6.1	6.5
NH_2-Leucine	3.5	5.3	3.3	7.3
Boc-Leucine	1.0	2.9	4.8	6.8
Boc-Leucine-Lysine	3.7	6.3	6.5	14.9
NH_2-Leucine Lysine	46.2	>50	>50	>50
Boc-Norleucine	1.0	1.5	2.6	3.6
NH_2-a-Aib Cer	4.2	10.4	5.8	11.7
a-Aib-Boc-Phealanine	11.6	12.7	10.9	14.7
a-Aib-Boc-D-Homophenyala-nine	6.1	11.6	9.8	13.6
NH_2-Dab(Boc)	3.9	7.3	9.2	6.8
NH_2-Dap(Boc)	3.0	3.8	5.0	8.4

Table 8: Screening against MCF-7 and A549 tumor cell lines; the "+" sign indicates the activity at 50 μM concentration, whereas the "-" sign indicates relatively inactive.

Non-Peptidomimetic Ceramide	conc. MCF7/ A549
2-Chlorophenylacetyl	+/+
1-Phenyl-1-cyclopentanoyl	+/+
1-Phenyl 1-cyclopropanoyl	+/+
t-Butylphenylthio	+/+
4-Methylphenyloxo	+/+
2-Nitrophenylpyruvic	+/+
2-Bromophenyl acetyl	+/+
(S)-2(Phenylcarbamoyloxy)propionic	-/+
3-Ethyl-3-methyl glutaric	-/-
Tolylsulfonyl	+/+
O-Methyl acetyl	-/-
O-Methyl Sulfonyl	-/+
(DL)-thioctyl	+/+
Tolylsulfonyl	+/+
(*R*)-3-Methyl-2-NitroMethyl-OPA	+/+
(2-Methylsulfonyl ethyl)	+/+
1,2,3-Triazolecarboxyl	+/+
(1-Methylcyclopropyl)	+/+
2-Bromopropanoyl (C3)	-/-
Cyanoacetyl (C2)	+/+
(*S*)-2-Methylbutyl (C4) C-10	-/-
2,2-di-Phenylpropanoyl (C3)	-/-
3-Nitropropanoyl (C-3)	+/+
(±)-2-Phenylpropanoyl (C-3)	+/+
t-Butylacetyl (C2)	+/+
2-Bromoacetyl-1,3-di-(2-Bromohexadexanoyl	-/-
2-Propylpentanoyl	+/+
4-Methylhippuric	+/+
3,3,3-Triphenylpropionoyl	+/+
2-Bromo-2-Methylpropanoyl	+/+
3-Methylhippuric	+/+
Peptidomimetic Ceramide	**conc. MCF7/ A549**
Boc-HistdineBom	+/+
Boc-Phenylglycine	+/+
NH_2-Phenylglycine	+/+
Fmoc-Phenylglycine (4-CH_2NH-Boc)	+/+
NH2-Phenylglycine (4CH_2 NH_2)	+/+
(*S*)-2-Methylbutanoyl (C4) Alanine	-/+
Boc-D-Homophenylalanine	-/+
(*S*)-2-Methylbutyl Phenylglycine	-/-
NH_2-D-Homophenylalanine	-/+

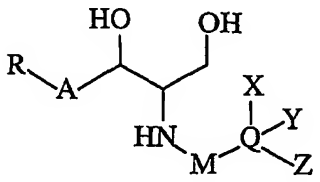
D-Homophenylalanine-Boc-Phenylalanine	-/-
NH_2-L-Phenylalanine (4-Phenyl)	+/+
Boc-Phenylalanine-[4-Nitro]	+/+
Boc- Phenylalanine [4-NH-Acetyl]	+/+
Boc- Phenylalanine (4-NH-Fmoc)	-/-
Boc-D-Phenylalanine-(4-Nitro)	+/+
Phenylalanine (4-NH-Fmoc)	-/-
Boc-Phenylalanine (4-NH_2)	+/+
Boc-Phenylaalanine (4-Nitro-tetrahydrofuryol)	+/+
Fmoc-Phenylalanine (4-CHO)	+/+
Fmoc-Phenylalanine (4-Gu$(Boc)_2$)	-/+
Fmoc-Phenylalanine (4-CH_2-OTrityl)	-/-
NH_2-Phenylalanine (4-CH_2-O-Trityl)	-/-
NH_2-Phenylalanine-(4Gu$(Boc)_2$)	+/+
Boc-Phenylalanine [4-NH-(4-Chloro-3-Nitro)-Benzenesulfonyl]	-/+
Boc-Phenylalanine [4-NH-(2-Bromobenz-enesulfonyl]	+/+
Boc-Phenylalanine (4-NH)-[(3-trifluoromethyl)-Benzenesulfonyl]-Ceramide	-/-
Boc-Phenylalanine (4-NH_2)	+/+
Boc-Phenylalanine [4-NH-(4-(trifluoromethoxy) benzenesulfonyl)]	-/-
Boc-Phenylalanine [4-NH-(2,4,6-Triisopropyl) benzenesulfonyl]	-/-
Boc-Phenylalanine [4-NH-(3-Furoic)]	+/+
2-Bromoacetyl-Phenylalanine	-/+
D-Homophenylalanine-Boc-D-Homophenylalanine	-/-
NH_2-Homophenylalanine	+/+
Boc-Tyrosine (Bromobenzyl)	-/-
NH_2-Homotyrosine (O-Trityl)	-/-
Fmoc-p-Tyrosine (O-^{t}Butyl)	-/+
N-Fmoc-m-Tyrosine (O-^{t}Butyl)	+/+
Boc-a-Abu	+/+
Boc-a-Aib	+/+
Cyclhexylalanine- Quinaldic	-/-
Fmoc-(c/t)Cyclohexylalanine-(4-NH-Boc)	-/-
Boc-O-Benzyl-Serine-leucine	+/+
NH_2-O-Benzyl-Serine-Leucine	-/-
Boc-Leucine-Lysine-OH	-/-
NH_2-Dab	-/-

Although the invention has been described with reference to specific examples and embodiments, those of ordinary skill in the art will readily recognize that various equivalent

elements may be substituted without departing from the spirit and scope of the invention defined in the appended claims.

What is claimed is:

1. A compound of the formula:



wherein:

R is C_nH_{2n+1}, where n is an integer of from 1-18;

A is CH_2-CH_2, CH_2-CH(OH), or cis, trans or cis+trans CH=CH;

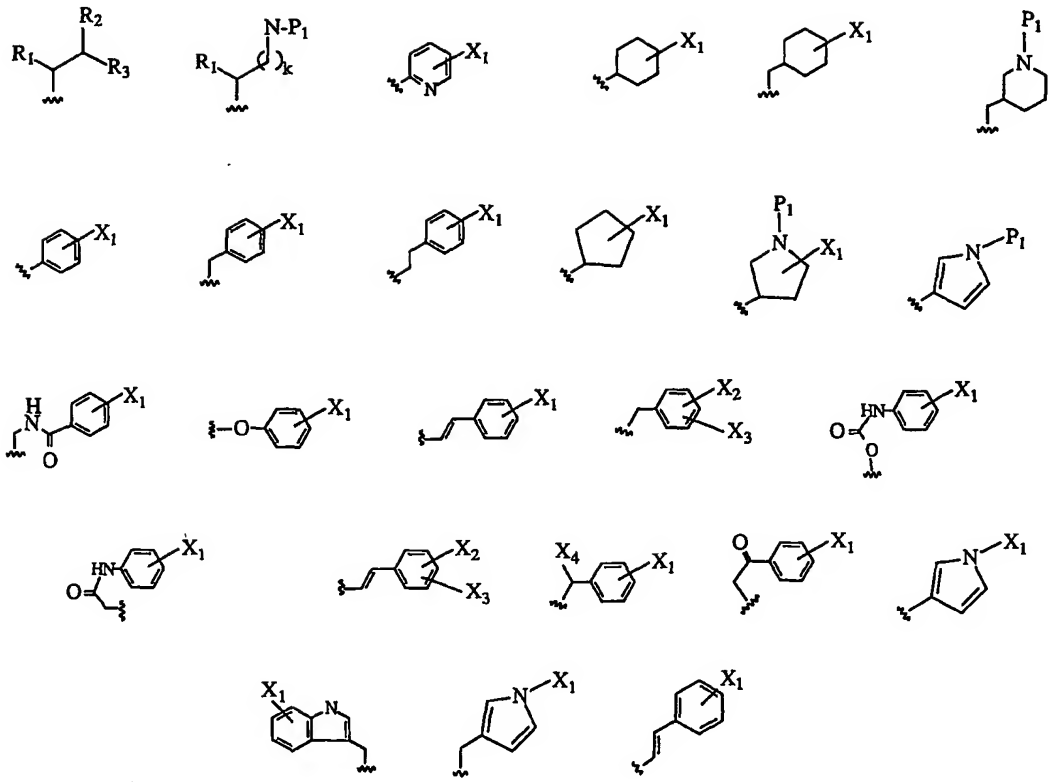
M is C(O) or CH_2;

Q is C, OC or $S(O_2)N$;

Y is H, OH, C_1-C_6 alkyl, C(O)OH, aryl, phenyl, NH_2, NO_2, C_6H_5, a halogen, NH-P_1, (O) or (S);

Z is H, C_6H_5, alkyl, NH_2, NH-P_1 or C(O)OH, wherein when Y is (O) or (S), Z is not present, and wherein when X, Y and Z are present as different moieties, the compound has an *R*, an *S*, or any combination of the *R* and *S* configurations about the α-carbon;

X is F, Cl, Br, I, C_6H_5, O-Si$(CH_3)_3$, O-Si$(C_4H_9)_3$, O-Si$(C_6H_5)_3$, C_1-C_{17} alkyl, -CH_2-O-X_1, or



k is an integer from 0 to 6;

R_1, R_2, and R_3 are each independently H, C_1-C_6 alkyl, C_1-C_6 alkenyl, C_2-C_6 cycloalkyl, or aryl;

X_1 is H, OH, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, C_2-C_6 cycloalkyl, aryl, phenyl, a halogen, NO_2, CN, C(O)H, C(O)OH, $C(O)OCH_3$, $COCH_3$, CH_2OH, NH_2, $NHCH_3$, $CONH_2$, $N(CH_2CH_2)_2Cl_2$, $B(OH)_2$, furyl, *O*-alkyl or aryl sulfonate;

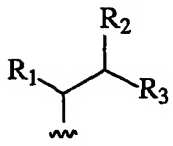
X_2 is –O-CH_2-, and X_3 is –O-CH_2- or –O-, such that X_2 and X_3 taken together form a heterocyclic ring; and

P_1 is H or an amino protecting group.

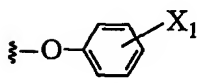
2. The compound of claim 1, wherein each of Y and Z is independently H or C_6H_5.

3. The compound of claim 1, wherein M is C(O), X is Br or a C_1-C_{17} alkyl group, Q is C, Z is H, C_6H_5, an alkyl group or C(O)OH.

4. The compound of claim 1, wherein M is C(O), and X is



5. The compound of claim 1, wherein M is C(O), X is

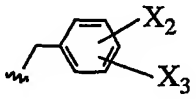


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, CH_3, NH_2, or NO_2; and

Z is H.

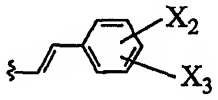
6. The compound of claim 1, wherein M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and

Z is H.

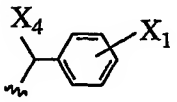
7. The compound of claim 1, wherein M is C(O), X is



Y is H, CH_3, NH_2, or NO_2; and

Z is H.

8. The compound of claim 1, wherein M is C(O), X is



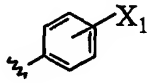
X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

X_4 is H, a halogen, OH, OCH_3, NO_2, CN, C_1-C_6 alkyl or C_2-C_6 cycloalkyl;

Y is H, CH_3, NH_2, or NO_2; and

Z is H.

9. The compound of claim 1, wherein M is C(O), X is

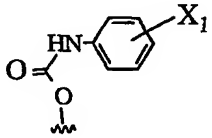


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

10. The compound of claim 1, wherein M is C(O), X is

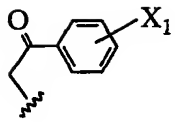


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

11. The compound of claim 1, wherein M is C(O), X is

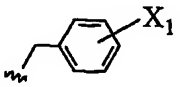


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H; and

Z is H, C_1-C_6 alkyl or C_2-C_6 cycloalkyl.

12. The compound of claim 1, wherein M is C(O), X is

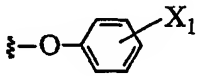


X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

13. The compound of claim 1, wherein M is C(O), X is



X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);

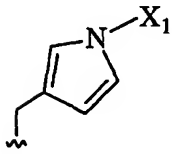
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

14. The compound of claim 1, wherein M is C(O); X is $-CH_2$-O-X_1;

X_1 is H, C_1-C_6 alkyl, F, Cl, Br, I, OH, OCH_3, NO_2, CN, C(O)OH, $C(O)OCH_3$, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, phenyl or aryl;

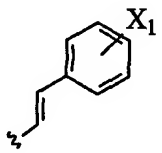
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

15. The compound of claim 1, wherein M is C(O), X is



X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;
Y is H, C_1-C_6 alkyl, aryl, phenyl, OH, C(O)OH, NH_2, NO_2, (O) or (S);
Z is H or C(O)OH, wherein when Y is (O) or (S), Z is not present.

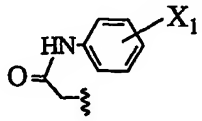
16. The compound of claim 1, wherein M is C(O), X is



X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl; and
Z is NH_2 or NH-P_1.

17. The compound of claim 1, wherein M is C(O), X is
X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl;
Y is H, OH, a halogen, NH_2, NH-P_1, (O) or (S).

18. The compound of claim 1, wherein M is C(O), X is



and X_1 is H, C_1-C_6 alkyl, C_1-C_6 *O*-alkyl, C_1-C_6 *S*-alkyl, $COCH_3$, CH_2OH, phenyl or aryl.

19. The compound of claim 1, wherein P_1 is selected from the group consisting of Boc, Fmoc, Troc, silyl, sulfonyl, acetyl and benzyl.

20. The compound of claim 1, wherein the compound has the D-*threo* configuration.

21. The compound of claim 1, wherein the compound has the L-*threo* configuration.

22. The compound of claim 1, wherein the compound has the D-*erythro* configuration.

23. The compound of claim 1, wherein the compound has the L-*erythro* configuration.

24. The compound of claim 3, wherein X is Br, Y is H and Z is H.

25. The compound of claim 24, wherein n is 11 and A is CH_2-CH_2.

Fig. 1A

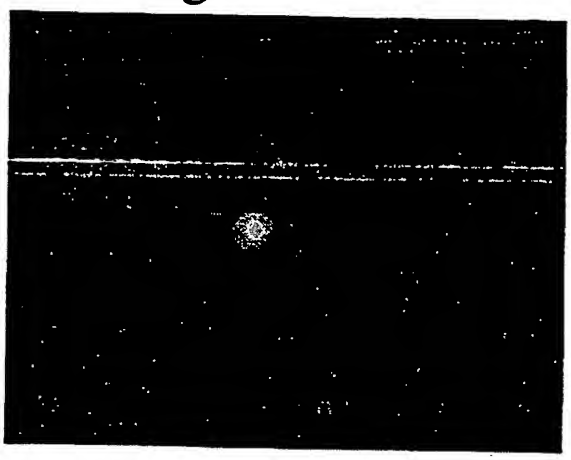


Fig. 1B

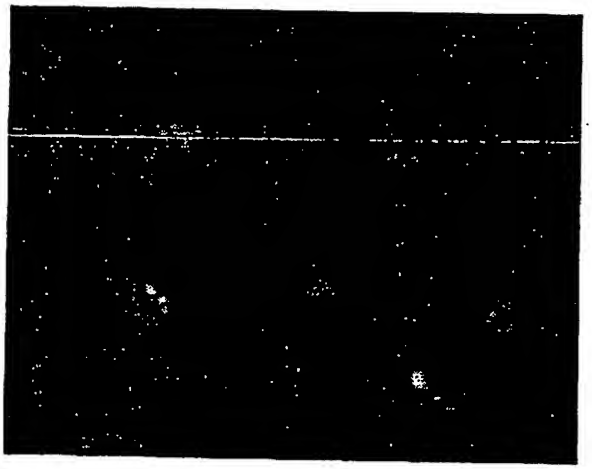


Fig. 1C

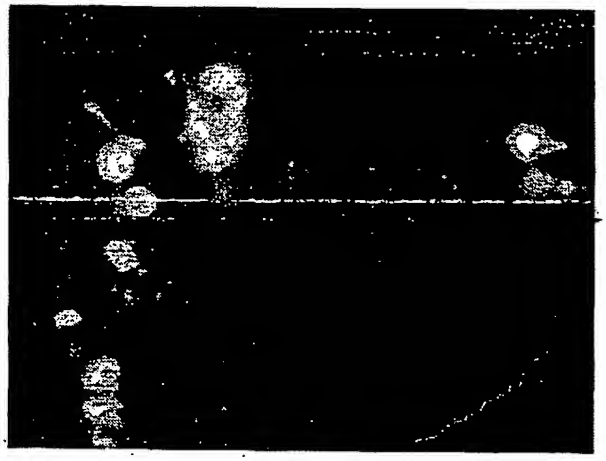


Fig. 1D

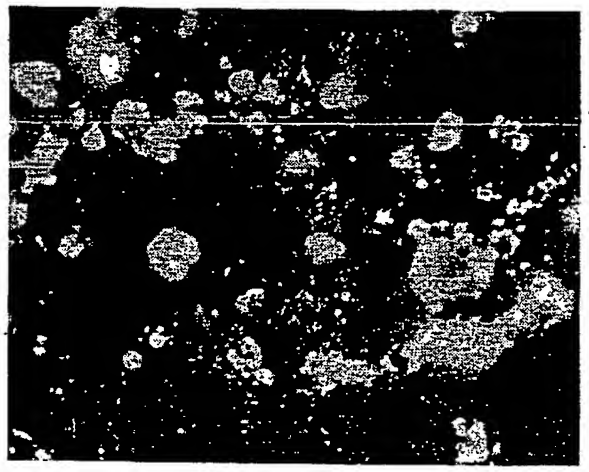


Fig. 1E

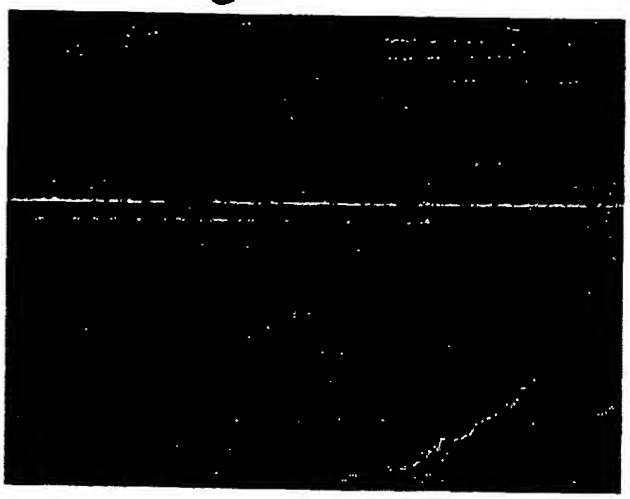


Fig. 1F

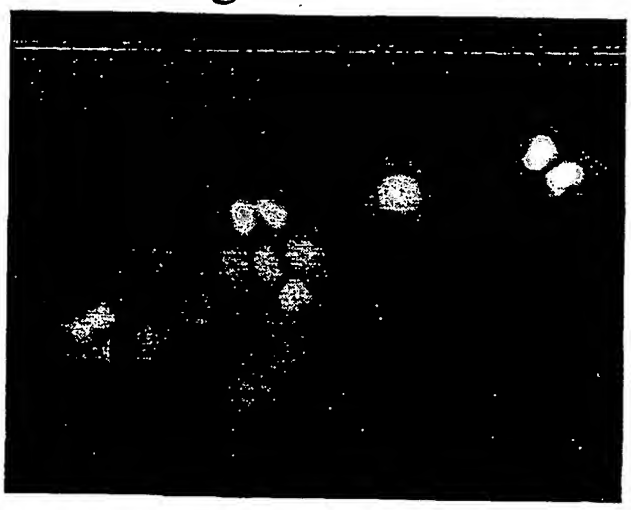


Fig. 1G

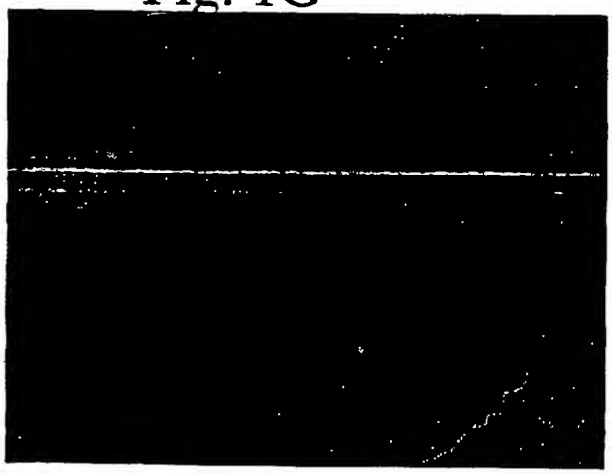


Fig. 1H

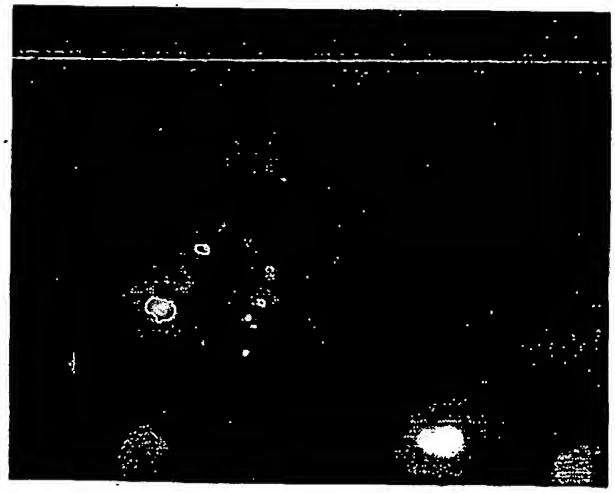
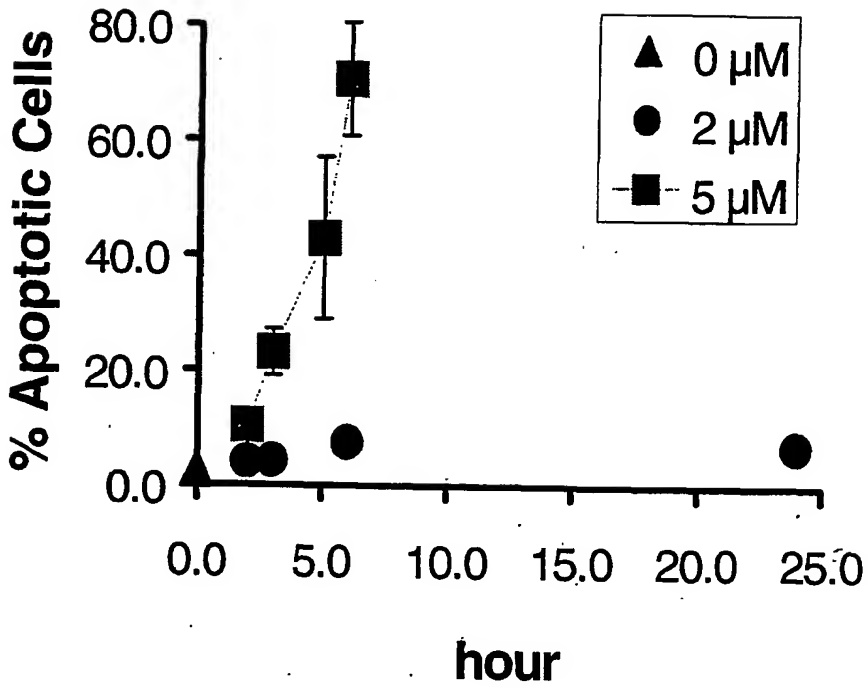


Fig. 2A.


% Apoptotic Cells
80.0
60.0
40.0
20.0
0.0
0.0
5.0
10.0
15.0
20.0
25.0
hour
0 μM
2 μM
5 μM

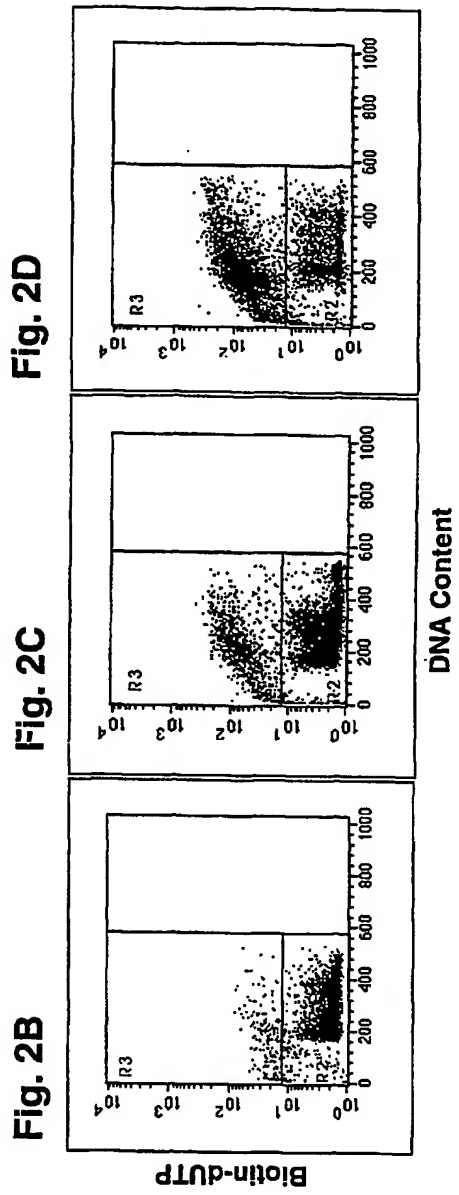


Fig. 2D
Fig. 2C
Fig. 2B
R3
R2
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
DNA Content
Biotin-dUTP

Fig. 3A

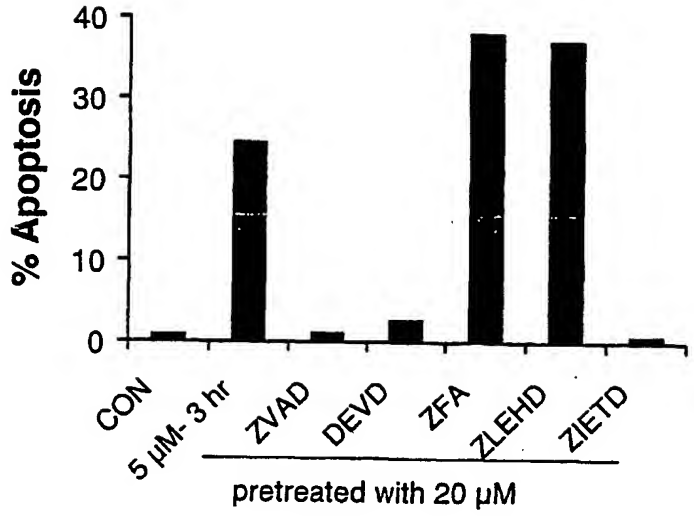

% Apoptosis
40
30
20
10
0
CON
5 μM- 3 hr
ZVAD
DEVD
ZFA
ZLEHD
ZIETD
pretreated with 20 μM

Fig. 3B

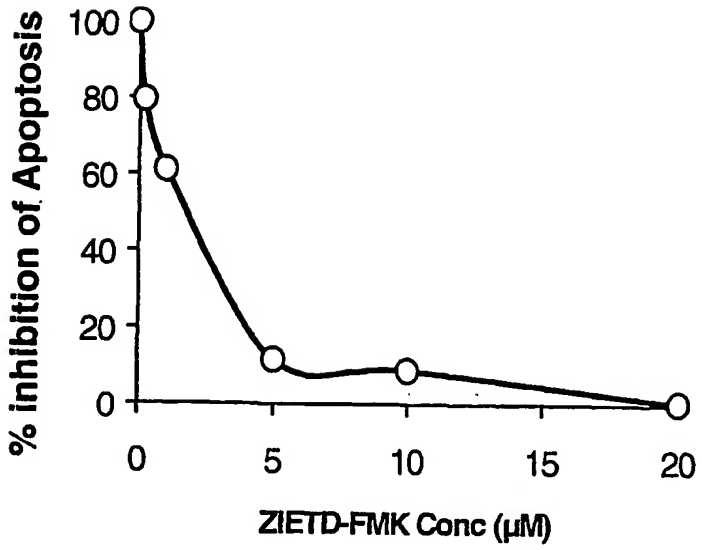

% inhibition of Apoptosis
100
80
60
40
20
0
0
5
10
15
20
ZIETD-FMK Conc (μM)

Fig. 4A

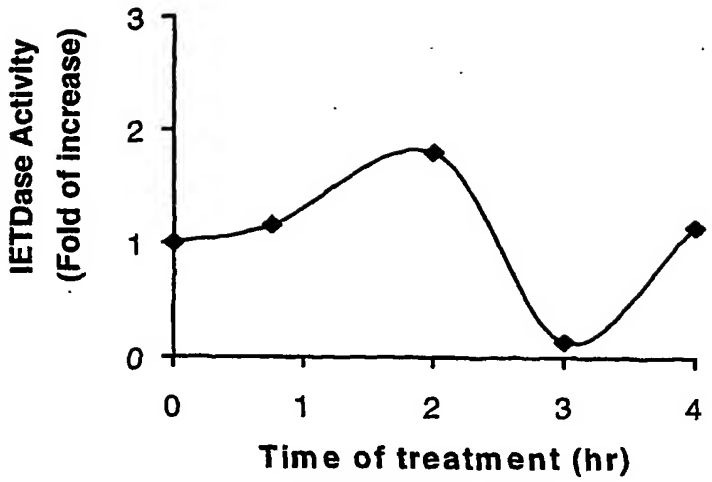
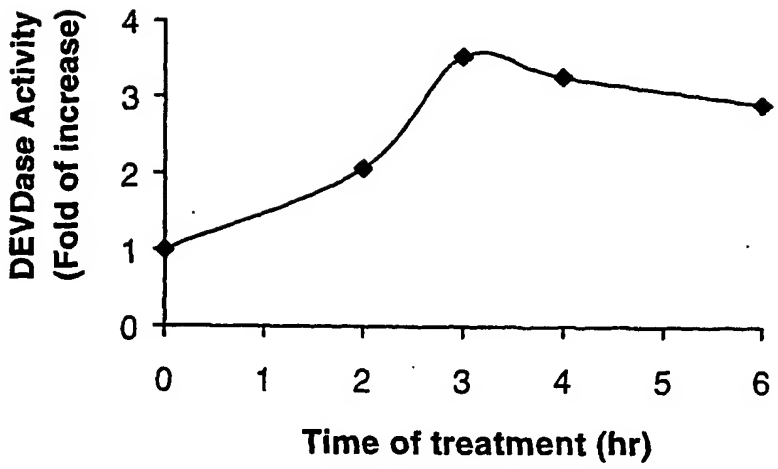

IETDase Activity
(Fold of increase)
3
2
1
0
0
1
2
3
4
Time of treatment (hr)

Fig. 4B


DEVDase Activity
(Fold of increase)
4
3
2
1
0
0
1
2
3
4
5
6
Time of treatment (hr)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/09894

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07C 321/00
US CL :554/66, 68, 69
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 554/66, 68, 69

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chem. abstr., Vol. 69, No.21, 18 November 1968 (Columbus, OH, USA) , page 8194, column 1, the abstract No. 87432p, WEISS et al. 'Synthesis of mono-, di, and tripeptidyl amides of dihydrosphingosine'. Journal of Chem. Eng. Data. 1968, 13(3), 450-1.	1-2, 6, 12
X	Chem. abstr., Vol. 131, No. 24, 13 December 1999 (Columbus, OH, USA), column 1, the abstract No. 131:327359q. LANZENDOERFER et al. 'Cosmetic and dermatologic compositions containing ceramides'. Eur. Pat. Appl. EP 955,038. 22 April 1999.	1-4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 JULY 2001	Date of mailing of the international search report 24 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DEBORAH D. CARR Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/09894

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chem. Abstr., Vol. 132, No. 17, 24 April 2000 (Columbus, OH, USA), page 1203, column 2, the abstract No. 227218v. ABE et al. 'Preparation and membrane characteristic of liposomes having ceramide 3'. Fragrance Journal, 1999, 27(10), 58-64 (Jap).	1-4
X	US 5,959,127 A (SEMERIA et al.) 28 September 1999, entire document.	1-2, 9
X	Chem. abstr., Vol. 116, No. 16, 20 April 1992 (Columbus, OH, USA), page 957, column 1, the abstract 165534d. HIRATA et al. '"Chemistry of succinimido esters. XXI. Determination of sphingosine bases as N-arylacetyl derivatives by normalphase HPLC (high performance liquid chromatography)'. (Natl. Chem. Lab. Ind., Tuskuba, Japan 305) 1991, 40(12), 1088-94.	1-2, 9